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1321191

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**APPLICATION NUMBER: 60/619,817**

**FILING DATE: *October 18, 2004***

**RELATED PCT APPLICATION NUMBER: *PCT/US05/09391***



Certified by

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and Director of the United States  
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22763 U.S. PTO

101804

PTO/SB/16 (04-04)

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# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

EV503463638 US

00746 U.S. PTO  
60/619817



101804

INVENTOR(S)						
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<u>James Richard</u>		<u>Eshleman</u>		<u>Lutherville, MD</u>		
Additional inventors are being named on the <u>one</u> separately numbered sheets attached hereto						
TITLE OF THE INVENTION (500 characters max)						
<u>Ligand-Sensitive Point Mutation Detection</u>						
Direct all correspondence to: CORRESPONDENCE ADDRESS						
<input type="checkbox"/> Customer Number: <span style="border: 1px solid black; display: inline-block; width: 200px; height: 30px; vertical-align: middle;"></span>						
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<input checked="" type="checkbox"/> Firm or Individual Name	<u>Johns Hopkins University</u>					
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ENCLOSED APPLICATION PARTS (check all that apply)						
<input checked="" type="checkbox"/> Specification Number of Pages	<u>59</u>		<input type="checkbox"/> CD(s), Number	<u></u>		
<input type="checkbox"/> Drawing(s) Number of Sheets	<u></u>		<input type="checkbox"/> Other (specify)	<u></u>		
<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76						
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT						
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.			FILING FEE Amount (\$)			
<input type="checkbox"/> A check or money order is enclosed to cover the filing fees.			<div style="border: 1px solid black; width: 100px; height: 50px; text-align: center; margin: 0 auto;">\$80.00</div>			
<input type="checkbox"/> The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: <u></u>						
<input checked="" type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.						
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.						
<input type="checkbox"/> No.						
<input checked="" type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: <u>HDO429645, A1467465, A1 CA081439, A1048854</u>						

[Page 1 of 2]

Respectfully submitted,

SIGNATURE

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Date

18-OCT-04

REGISTRATION NO.

55,601

(if appropriate)

Docket Number:

4547

## USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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**PROVISIONAL APPLICATION COVER SHEET**  
**Additional Page**

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Docket Number 4547

INVENTOR(S)/APPLICANT(S)		
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Chanjuan	Shi	Baltimore, MD
Susan Henrietta	Eshleman	Lutherville, MD

[Page 2 of 2]

Number 2 of 2

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**CERTIFICATE OF EXPRESS MAILING**

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18-Oct-04

DATE of Signature  
And of Mail Deposit

Cheryl Rissold

Signature

**U.S. Provisional Patent Application**

**JHU Ref. No. DM-4547**

**LigAmp: Sensitive Point Mutation Detection**

**Inventors:  
James Eshleman  
Chanjuan Shi  
Susan Eshleman**

All publications, patents and patent applications disclosed herein are incorporated into this application by reference in their entirety.

For example: "Sambrook et al, Molecular Cloning, A Laboratory Manual (volumes I-III) 1989, Cold Spring Harbor Laboratory Press, USA" and "Harlowe and Lane, Antibodies a Laboratory Manual 1988 and 1998, Cold Spring Harbor Laboratory Press, USA" provide sections describing methodology for antibody generation and purification, diagnostic platforms, cloning procedures, etc. that may be used in the practice of the instant invention.

The following claim(s) of this provisional application are not to be construed as limiting the disclosed invention(s). The claim(s) are included for compliance with patent application structural regulations that may be imposed by international patent offices.

We claim:

1. A method of detecting mutations in DNA comprising converting a single base substitution into a completely foreign molecule.

## Report of Invention Disclosure Form (ROI)

This form is to be completed and submitted to the JHU office of Licensing and Technology Development (LTD) by anyone who believes they have developed a new invention. The purpose of this form is to enable LTD to evaluate whether legal protection to the invention will be sought and/or commercialization pursued. Please submit this form with all inventor(s) and Department Director(s) signatures. Visit the LTD web site at <http://www.ltd.jhu.edu/ForHopkinsInventors/index.html> for .pdf and Word downloadable formats of this form.

## INVENTION INFORMATION

**Title of Invention:** [Title should be sufficiently descriptive to identify the invention yet not reveal unique unpublished details.]

LigAmp: Sensitive Point Mutation Detection

**Name of Lead Inventor:**

Eshleman	James	Richard	MD, PhD
Last	First	Middle	Degree

**Lead Inventor Information:** [The Lead Inventor should be a full time JHU faculty member. He/She will be the primary contact person for LTD on all matters associated with this Report of Invention, including processing, patent prosecution and licensing. For reasons of administrative efficiency, it is the responsibility of the Lead Inventor to keep all other JHU inventors named on this Report of Invention informed of the status of such matters.]

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Are you a Kennedy Krieger Institute employee or investigator? ☐ Yes ☒ No

Additional inventors: ☒ Yes ☐ No If yes, please complete Additional Inventors section for each inventor.

**LTD Internal Use Only:** REF-4547 TLA GHS Field of Use 2A



**ADDITIONAL INVENTOR(S)**

Please copy this page for additional inventors as necessary

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Are you a Kennedy Krieger Institute employee or investigator? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No				

## INVENTION DESCRIPTION

Describe the invention completely, using the outline given below. Please provide an **electronic copy** of the invention disclosure document, references, and abstracts in Windows format on CD-ROM or floppy disk if possible

### 1. Marketing Summary [Please provide a non-confidential summary of the invention that can be used for marketing purposes. Unique details that are published may also be included.]

We have developed a new strategy that permits detection of small amounts of point mutation containing DNA in the presence of an excess amount of wild-type DNA (e.g. 1:10,000 to 1:100,000). This is accomplished by first performing selective oligonucleotide ligation on the mutation containing DNA using two unique DNA primers. The DNA primers contain regions that are target specific, but also contain tails (e.g. M13) that permit subsequent amplification of the ligated product. Since the strategy involves a ligation step followed by a real-time PCR amplification step, we have designated it as "LigAmp". One of the ligation primers also contains a region of completely foreign DNA (e.g. lac-Z) that is used as probe site during the subsequent RQ-PCR (real time quantitative PCR) step. High selectivity is achieved since probe cleavage cannot occur without prior ligation. The ligation step can be multiplexed to simultaneously detect multiple point mutations.

Applications to Cancer, Infectious Disease and Genetics testing are described below.

**SOFTWARE** –Does this disclosure include a software element or software is implemented in the invention

☐ Yes ☒ No

If yes, please complete the Software Information Form which can be found at: \_\_\_\_\_

**BIOLOGICAL MATERIAL** – Does this disclosure include biological material,

☐ Yes ☒ No

If yes, please attach a list of materials for reference. A Tangible Property Report of Invention form may be completed if the disclosure is biological materials only. You can find this form at: [http://www.ltd.jhu.edu/For\\_Hopkins\\_Inventors/reporting.html](http://www.ltd.jhu.edu/For_Hopkins_Inventors/reporting.html)

### 2. Problem Solved [Describe the problem solved by this invention]

A major problem in molecular analysis without current adequate solution is the accurate and sensitive detection of single base change containing DNA (base substitution mutations, SNPs, etc).

Other methods to detect minor components of a DNA mixture (e.g. oligonucleotide ligation assay (OLA), allele-specific PCR (AS-PCR), amplification refractory mutation system (ARMS), standard realtime PCR (Q-PCR or RQ-PCR), among others) exist, however the limit of detection for most of these methods is currently only between 1:10 and 1:100, which is insufficient for many applications.

This new invention permits relatively simple quantitative detection of small amounts of single base change containing molecules using a highly accurate method of detection and permitting a limit of detection of approximately 1:10,000 to 1:100,000, and possibly greater.

**3. Novelty** [Identify those elements of the invention that are new when compared to the current state of the art]

This method uses a modification of Oligonucleotide Ligation Assay (OLA) to convert DNA molecules with only single base changes to those which contain a completely unique stretch of DNA (about 20 bases for use as probe detection). In the second part of the assay, realtime quantitative PCR (Q-PCR) is performed using universal primers to sensitively and quantitatively detect the probe containing DNA (and therefore the single base change). Because the strategy involves a ligation step, followed by a Q-PCR amplification step, we have designated the method as "LigAmp".

OLA typically involves PCR amplification followed by ligation and detection of ligated products. LigAmp involves use of ligase to convert the point mutation to a foreign DNA molecule, which is then detected using real time PCR or other amplification strategy.

This method can be used for early detection of cancer, cancer minimal residual disease testing, HIV drug-resistant minority variant detection, identification of samples containing rare alleles and bone marrow transplantation engraftment monitoring.

**4. Potential Commercial Use** – [What products can be produced with this invention.]

LigAmp permits sensitive detection of point mutations and can be multiplexed.

Kits could be produced for:

Early detection of Cancers (if cancers are detected early, patients can undergo definitive curative surgery)

Cancer Minimal residual disease testing (e.g. determining the whether the cancer has been eliminated or is still present, following surgery or chemotherapy, etc). Such kits could also be used to identify if a cancer is coming back (so called "molecular relapse").

Infectious disease minority variant detection: viral (e.g. HIV, HBV, HCV, etc) minority variant detection (conferring anti-viral drug resistance) and other anti-microbial resistance testing where point mutations are the basis of the resistance (e.g. mycobacterial infections).

Human genetics applications include: detection of panels of point mutations such as those in cystic fibrosis. One could produce an assay to simultaneously detect pro-coagulation mutations, or cardiovascular risk assessment, or a panel of SNPs for pharmacogenomics of forensic testing. In one format of the assay, one could simply determine whether a patient is wild type or carries a mutation. Additionally, one could detect known parental point mutations in fetuses from the peripheral blood of known wild-type mothers, without the need for amniocentesis.

**5. Commercialization** - List any companies that you feel may be interested in this technology or are doing similar research. Indicate how the invention complements the company's existing technology. If known, provide the names of any companies (and a contact person) that have contacted you regarding your research related to the invention.

☐ No company interest known at this time.

**Keywords – Please circle the categories and keywords that accurately describe the present invention.**

**CHEMICAL**

- ☐ Additives
- ☐ Alternative Energy
- ☐ Antioxidants
- ☐ Batteries
- ☐ Catalyst
- ☐ Coal Conversion
- ☐ Coatings
- ☐ Effluent Treatment
- ☐ Elastimers
- ☐ Electrochemistry
- ☐ Exhaust Treatment
- ☐ Foams
- ☐ Food Chemistry
- ☐ Fuel Cells
- ☐ Gas Conversion
- ☐ Gels
- ☐ Monomers
- ☐ Oxidation
- ☐ Petroleum
- ☐ Photochemistry
- ☐ Polymers
- ☐ Remediation
- ☐ Solvents

**DIAGNOSTIC**

- ☐ Antibody
- ☒ Assay
- ☐ Biochip
- ☐ Contrast Agent
- ☒ Detection
- ☒ DNA Probe
- ☐ Elisa
- ☐ Imaging
- ☐ Immunoassay
- ☐ In Situ
- ☒ Marker
- ☒ Measurement
- ☐ MRI
- ☐ Point of Use
- ☐ Radioisotope
- ☐ Transgenic
- ☐ Ultrasound

**GENOMICS**

- ☒ Allele
- ☐ Bioinformatic
- ☒ cDNA
- ☒ Epidemiology
- ☐ EST
- ☒ Gene
- ☒ Homologue
- ☐ Isogene
- ☐ Library
- ☒ Mutation
- ☒ Pharmacogenomics
- ☒ Polymorphism
- ☐ Positional Cloning
- ☐ Proteomics
- ☐ Receptor
- ☐ RNA
- ☐ Target Validation

**MEDICAL DEVICE**

- ☐ Delivery
- ☒ Diagnosis
- ☐ Imaging
- ☒ Measurement
- ☐ Optical
- ☐ Safety
- ☐ Surgical
- ☐ Treatment

**RESEARCH TOOL**

- ☐ Animal Model
- ☐ Antibody
- ☐ Cell Line
- ☐ Culture
- ☒ Directed Evolution
- ☐ DNA Probe
- ☐ DNA/RNA Sequencing
- ☐ DNA/RNA Synthesis
- ☐ Electrophoresis
- ☐ Elisa
- ☐ Enzyme
- ☐ Equipment
- ☐ Expression System

- ☐ Immunoassay
- ☐ Label
- ☒ PCR
- ☐ Protein Sequencing
- ☐ Protein Synthesis
- ☐ Reagent
- ☐ Spectroscopy
- ☐ Tissue Culture
- ☐ Vector

**SCREENING**

- ☒ Assay
- ☐ Biochip
- ☐ Combinatorial Biology
- ☐ Combinatorial Chemistry
- ☒ Detection
- ☐ HTS
- ☐ Phage Display
- ☐ Screen
- ☐ Target

**THERAPEUTIC**

- ☐ Analgesic
- ☐ Anesthetic
- ☐ Angiogenesis
- ☐ Antibiotic
- ☐ Antibody
- ☐ Antifungal
- ☐ Antiinflammatory
- ☐ Antisense
- ☐ Antiviral
- ☐ Apoptosis
- ☐ Cell Signaling
- ☐ Cell Therapy
- ☐ Disease Model
- ☐ Drug Delivery
- ☐ Drug Design
- ☐ Fertility
- ☐ Gene Therapy
- ☐ Hormone
- ☐ Immunotherapy
- ☐ Natural Product
- ☐ Peptides

- ☐ Pro-drug
- ☐ Proteins
- ☐ Small Molecule
- ☐ Tissue Engineering
- ☐ Transplant
- ☐ Vaccine
- ☐ Virus
- ☐ Wound Healing

**DISEASES**

- ☐ Aging
- ☒ Blood
- ☒ Cancer
- ☒ Cardiovascular
- ☐ Dermatologic
- ☐ Endocrine
- ☐ Gastrointestinal
- ☐ Genitourinary
- ☐ Hepatic
- ☐ Immune
- ☐ Infectious
- ☐ Metabolic
- ☐ Musculoskeletal
- ☐ Neurological
- ☐ ObGyn
- ☐ Ophthalmological
- ☐ Oral
- ☐ Pediatric
- ☐ Psychiatric
- ☐ Respiratory

**ADDITIONAL KEY WORDS:**

**STAGE OF DEVELOPMENT**

- ☐ Unspecified
- ☒ Discovery
- ☒ Preclinical
- ☐ Prototype
- ☐ Phase I
- ☐ Phase II
- ☐ Phase III
- ☐ NCE

**7. Detailed Description of the invention -** On a separate page(s), attach a detailed description of how to make and use the invention. The description must contain sufficient detail so that one skilled in the same discipline could reproduce the invention. Include the following as necessary:

- 1- data pertaining to the invention;
- 2- drawings or photographs illustrating the invention;
- 3- structural formulae if a chemical;
- 4- procedural steps if a process;
- 5- a description of any prototype or working model;

In general, a manuscript that has been prepared for submission to a journal will satisfy this requirement.

**8. Workable Extent/Scope** [Describe the future course of related work, and possible variations of the present invention in terms of the broadest scope expected to be operable; if a *compound*, describe substitutions, breadth of substituents, derivatives, salts etc., if *DNA or other biological material*, describe modifications that are expected to be operable, if a *machine or device*, describe operational parameters of the device or a component thereof, including alternative structures for performing the various functions of the machine or device]

This is a general method with a wide range of potential applications. The major features are sensitive detection of genetic changes, including point mutations, and the ability to multiplex their detection.

Adapting the system to detect different targets is accomplished by simply designing new oligonucleotides for the first step. We have demonstrated proof-of-principle experiments for the following:

- Early detection of cancer

- Minority variant HIV detection (K103N etc).

Future related work will further expand the range of applications, including but not limited to: SNP/rare allele detection in pooled DNA, detection of known paternal point mutations in maternal peripheral blood, and bone marrow engraftment analysis.

The second step of the process is universal. M13 forward and reverse primers are used in addition to two probes with no cross-reactivity to each other. Therefore one can easily adapt the system to different DNA targets to determine their absolute or relative concentrations. Other primers can be used as universal primers and any region of foreign DNA can be used for the detection probe. Detection of the product is most commonly done by real-time PCR. Alternative methods of detection include bead hybridization, rolling circle amplification, etc.

**9. References** [Please cite relevant journal citations, patents, general knowledge or other public information related to the invention and distinguish between references that (A) contain a description of the current invention from those that (B) contains background information.]

Shi C, Eshleman SH, Jones D, Fukushima N, Hua L, Parker AR, Yeo CJ, Hruban RH, Goggins MG, and Eshleman JR. LigAmp: Sensitive Detection of Single Nucleotide Differences. *Nature Methods*, In Press.  
Flys T, Nissley DV, Claasen CW, Jones D, Shi C, Guay LA, Musoke P, Mmimo F, Strathern JN, Jackson JB, Eshleman JR and Eshleman SH. Sensitive drug resistance assays reveal long-term persistence of HIV-1 variants with the K103 nevirapine (NVP) resistance mutations in some women and infants after single dose NVP: HIVNET 012. In Preparation.

#### LigAmp vs. OLA

Oligonucleotide Ligation Assay involves selective ligation of ligation primers on a mutation containing DNA target. The DNA target is most commonly produced by PCR amplification, and the ligated products are commonly detected on an automated DNA sequencing instrument. LigAmp involves selective ligation to convert DNA into a foreign molecule that can be subsequently detected by an amplification strategy, most commonly real-time PCR.

Redston, M.S., Papadopoulos, N., Caldas, C., Kinzler, K.W. & Kern, S.E. Common occurrence of APC and K-ras gene mutations in the spectrum of colitis-associated neoplasias. *Gastroenterology* **108**, 383-92 (1995).

Rothschild, C.B., Brewer, C.S., Loggie, B., Beard, G.A. & Triscott, M.X. Detection of colorectal cancer K-ras mutations using a simplified oligonucleotide ligation assay. *J Immunol Methods* **206**, 11-9 (1997).

#### LigAmp vs. LCR

Ligase chain reaction is an assay that also uses selective ligation. Ligase is substituted for the polymerase that is used in PCR and four oligonucleotides are used (2 per DNA strand).

Barany F. Genetic disease detection and DNA amplification using cloned thermostable ligase. *PNAS*, **88**: 189-193 (1991).

**Sensitive drug resistance assays reveal long-term persistence of HIV-1 variants with the K103N nevirapine (NVP) resistance mutation in some women and infants after single dose NVP: HIVNET 012**

Tamara Flys<sup>1</sup>, Dwight V. Nissley<sup>2,3</sup>, Cassidy W. Claasen<sup>3</sup>, Dana Jones<sup>1</sup>, Chanjuan Shi<sup>1</sup>, Laura A Guay<sup>1</sup>, Philippa Musoke<sup>4</sup>, Francis Mmro<sup>4</sup>, Jeffrey N. Strathern<sup>3</sup>, J. Brooks Jackson<sup>1</sup>, James R. Eshleman<sup>1</sup>, and Susan H. Eshleman<sup>1\*</sup>

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Running Head: NVP resistance in HIVNET 012

**Sources of support:**

This work was supported by (1) the HIV Network for Prevention Trials (HIVNET) and sponsored by the U.S. National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Dept. of Health and Human Services (DHHS), through contract N01-AI-35173 with Family Health International, contract N01-AI-45200 with Fred Hutchinson Cancer Research Center, and subcontracts with Makerere Univ. (NOI-AI-35173-417), (2) the HIV Prevention Trials Network (HPTN) sponsored by the NIAID, National Institutes of Child Health and Human Development (NICHD), National Institute on Drug Abuse, National Institute of Mental Health, and Office of AIDS Research, of the NIH, DHHS (U01-AI-46745 and U01-AI-48054), (3) the Adult AIDS Clinical Trials Groups (NIH, Division of AIDS, NIAID, U01-AI-38858), and (4) R01-HD042965-01.

This publication has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. NO1-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

## **ABSTRACT**

**Objective:** The HIVNET 012 trial showed that single dose (SD) nevirapine (NVP) can prevent HIV-1 mother-to-child transmission. However, NVP-resistant variants were selected in some women and infants. We tested whether variants with the K103N mutation could persist at low levels in women and infants for a year or more after SD NVP exposure using an assay with a lower limit of detection of 0.1%.

**Methods:** Plasma collected before and up to 12-24 months after SD NVP was available from nine women and five infants in the HIVNET 012 cohort. Samples were genotyped using the ViroSeq system, and the level of K103N-containing variants was quantified using a sensitive resistance assay, LigAmp. Selected samples were also analyzed with a yeast-based, phenotypic resistance assay, TyHRT.

**Results:** At 6-8 weeks after NVP, K103N was detected by ViroSeq in eight of 9 women and two of 5 infants, and was detected by LigAmp at a level above 0.1% in eight of 9 women (median=12.6%) and three of 5 infants (median=0.55%). At 12-24 months, K103N was not detected by ViroSeq in any of the samples, but was detected above pre-NVP levels in three of 9 women (at 0.8%, 1.3%, and 3.5%) and one of 5 infants (at 1.5%). Persistence of K103N in the 12-24 month samples was confirmed using the TyHRT assay.

**Conclusions:** K103N-containing HIV-1 variants can persist in some women and infants for a year or more after SD NVP. Assays that can quantify HIV-1 variants at low levels may provide new insight into the impact of antiretroviral drug exposure on HIV-1 evolution. Further studies are needed to determine the clinical significance of minority variants with NVPR mutations.

**Key words:** HIV-1, resistance, nevirapine, minority variants, mother-to-child transmission, Uganda



## **INTRODUCTION**

Approximately 800,000 children are infected with HIV-1 world-wide, most in resource-poor settings that have limited access to antiretroviral drugs for treatment and prevention of HIV-1 infection. Short regimens of antiretroviral prophylaxis can reduce the risk of HIV-1 mother-to-child transmission. The HIVNET 012 regimen, which consists of a single dose of nevirapine (NVP) to women in labor and a single dose of NVP to infants shortly after birth, is simple, safe, inexpensive, and effective for prevention of mother-to-child transmission (pMTCT) [1,2]. The HIVNET 012 regimen is endorsed by the World Health Organization for use in resource-limited settings.

A potential disadvantage of the HIVNET 012 regimen is the emergence of NVP resistance (NVPR) in some women and infants after NVP administration. Women enrolled in HIVNET 012 were antiretroviral drug naive, and did not receive any other antiretroviral therapy, consistent with the standard of care in Uganda at the time the trial was performed. Six to eight weeks after single dose NVP administration, NVP-resistant HIV-1 strains were detected in 70 (25%) of 279 women [3] and 11 (46%) of 24 infants [4]. The most common NVPR mutations detected were K103N in women and Y181C in infants, although other NVPR mutations were also detected. Emergence of NVPR after single dose NVP was subsequently observed in other studies [5-10]. Selection of NVPR in this setting is concerning, since it could potentially reduce the efficacy of NVP-containing regimens for pMTCT in subsequent pregnancies, or the efficacy of non-nucleoside reverse transcriptase (RT) inhibitor (NNRTI)-containing regimens for future treatment of HIV-1 infection in women and infants who received NVP prophylaxis. Furthermore, a recent study from Thailand demonstrated that women with prior exposure to single dose NVP had a reduced virologic response to a subsequent NNRTI-containing regimen. The effect,

although slight, was more marked among women who received single dose NVP less than 6 months prior to treatment [11]. NVP-resistant variants selected in women by single dose NVP could also potentially be transmitted to infants by breast-feeding, or to others in the community.

Few studies have evaluated how long NVP-resistant HIV-1 variants persist in women or infants after single dose NVP. In HIVNET 012, long-term follow-up samples (from 12-24 months after single dose NVP) were available for 11 women and 6 infants who had NVPR at 6-8 weeks. No NVPR mutations were detected in those samples [4]. In the HIVNET 023 trial in Zimbabwe, variants with NVPR mutations faded from detection in all but one woman by 6 months post-partum [5]. However, in a South African cohort, 55/155 (35%) of women still had detectable NVPR mutations at 6 months post-partum (all with K103N, and two with G190A) [12]. Persistence of NVPR mutations in that study was associated with higher viral loads and lower CD4 cell counts. While those studies suggest that NVPR mutations are replaced with wild type HIV-1 over time in most women and infants, the genotyping assays used in those studies, which are based on population (bulk) sequencing, do not provide information about the level of NVP-resistant HIV-1 variants in plasma, and are relatively insensitive for detection of drug resistance mutations in HIV-1 variants that are present as mixtures with wild type virus.

In this exploratory study, we used sensitive and quantitative assays to analyze the emergence and persistence of HIV-1 variants with the K103N mutation in a subset of women and infants in the HIVNET 012 cohort.

## **MATERIALS AND METHODS**

### **HIV Genotyping**

HIV-1 genotyping was performed with the ViroSeq™ HIV-1 Genotyping System (Celera Diagnostics, Alameda, CA). This system involves HIV-1 RNA isolation and reverse transcription, followed by a single 40-cycle PCR amplification which yields a 1.8 kb PCR product. The PCR product is purified and sequenced with seven different primers using an ABI PRISM 3100 Genetic Analyzer (BigDye®, Applied Biosystems, Foster City, CA). Sequences were examined for mutations associated with NVPR (A98G, L100I, K101E/P/Q, K103N/S, V106A/M, V108I, V179D, Y181C/I, Y188C/H/L, G190A/S/E, M230L), as well as accessory mutations (V106I, P225H, Y318F) and mutations associated with NVP hypersusceptibility (P236L) [IAS-USA Drug Resistance Mutations Group (2002), Stanford HIV RT and Protease Sequence Database (<http://hivdb.stanford.edu/cgi-bin/NNRTIResiNote.cgi>)]. Polymorphisms at positions 135 and 283, which may also influence NVP susceptibility [13], were also noted.

### **Quality control of sequencing data**

For each sample, a sequence corresponding to protease amino acids 1-99 and RT amino acids 1-320 was obtained. Genotypes were analyzed only if bi-directional sequence data was obtained at all positions of NVPR mutations. Phylogenetic reconstructions were performed using the complete set of sequences obtained from the maternal and infant samples. This analysis provided evidence that the data set from those subjects was valid, without evidence of sample mis-identification or cross-contamination.

### **Detection of the K103N mutation using the LigAmp assay**

#### **Oligonucleotide ligation**

Ligation was performed using 100 pg of plasmid-derived or plasma-derived ViroSeq

PCR products amplified without dUTP. The concentration of DNA in each sample was determined using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE). Gel-purified ligation oligonucleotides were purchased from Invitrogen, Corp. (Carlsbad, CA, Table 1). PCR products (ligation templates) were incubated with 1 pmol of the upstream oligonucleotide, 2 pmol of the downstream oligonucleotide and 4 U *Pfu* DNA ligase in 1x *Pfu* Ligase Buffer (Stratagene, La Jolla, CA). Samples were denatured at 95°C for 1 minute, followed by 99 two-step cycles alternating 95°C for 30 seconds with 50°C for 4 minutes. A negative control (no DNA template) was included with each set of ligation reactions, and was carried through the real-time detection step.

#### Real-time PCR Detection

Real-time PCR was performed using a SmartCycler (Cepheid, Sunnyvale, CA). Each 25 µl reaction contained 5 pmol M13 forward primer and 5 pmol M13 reverse primer (Invitrogen, Table 1), 5 µl of the unpurified ligation reaction, 12.5 µl platinum Quantitative PCR SuperMix-UDG (Invitrogen), and 5 pmol of the *LacZ* probe (Integrated DNA Technology, Coralville, IA, Table 1). Reactions were pre-incubated at 50°C for 2 min and 95°C for 2 min, followed by 45 two-step cycles of 95°C for 10 seconds alternating with 64°C for 25 seconds. The cycle threshold was manually set in the middle of the linear range of the amplification curve for each experiment (log scale). A negative control (no DNA template) was included with each set of real-time PCR reactions.

#### **Yeast TyHRT assay**

Reverse transcription assays were carried out in yeast strain DG1251 (*MATa ura3-167 trp1-hisG spi3-101 his3D200*). The plasmid pHART1 has the BsrGI-PvuII fragment of the HIV-1 RT coding removed resulting in the deletion of HIV-1 RT amino acids 37-250. BsrGI/PvuII linearized, gel purified pHART1 (100-200 ng) and HIV-1 RT domain PCR product (200-500 ng) were co-transformed into DG1251 using a lithium acetate protocol [14]. Transformants were selected on SC-URA (synthetic complete media missing uracil) + glucose plates [15]. Transformants were arrayed in small (100/plate) or large (25/plate) patches on SC-URA+glucose plates using sterile toothpicks. Following incubation overnight at 30°C arrayed patches were replica plated with velveteen to SC-URA+galactose plates with or without inhibitor and grown 2 days at 30°C to induce expression of the hybrid retroelements which are under the control of the *GALI* promoter [16]. The plates were then replica plated to SC-HIS (SC missing histidine) +/- inhibitor and grown for 3-4 days at 30°C to select reverse transcription events. NVP was dissolved in dimethylsulfoxide (DMSO) and used at 80-160  $\mu$ M at a final DMSO concentration of 1%.

For DNA sequencing, HIV-1 RT domains in TyHRT elements were obtained by growing isolates overnight in 10 ml SC-URA at 30°C and preparing DNA with a glass bead/phenol extraction method. DNA was suspended in 50  $\mu$ l water and 0.5-1.0  $\mu$ l was used in a 50  $\mu$ l PCR reaction to amplify the RT domain. Amplification was carried out for 35 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 2 min) using primers A-35 (5'GAACCTCCGAGATCGAAGA3') and I1097 (5'GCACTGCCTCTGTTAATTGT3') resulting in a PCR product that includes the HIV-1 RT coding region for amino acids 1-367. PCR products were purified with the Qiaquick 96 PCR Purification Kit (Qiagen Sciences, Germantown, MD) and sequenced with internal primers B275 (5'AGACTTCTGGGAAGTTCAAT3'), 220F (5'TGGAGAAAATTAGTAGATTT3') and J801 (5'ATCCCTGGGTAAATCTGACT3').

## **RESULTS**

### **Samples used for analysis**

Plasma samples were obtained from nine women and five infants enrolled in HIVNET 012 who had NVPR mutations detected using the ViroSeq assay 6-8 weeks after single dose NVP and who had pre-NVP samples and follow-up samples (collected 12-24 months after NVP administration) available for analysis. HIV-1 subtypes were determined previously by phylogenetic analysis of *pol* region sequences [3,17]. Three women and two infants had subtype A (M-660, M-790, M-847, I-466, I-545), and six women and three infants had subtype D (M-470, M-474, M-554, M-750, M-755, M-830, I-750, I-788, I-827). Samples were collected from the women prior to NVP, at delivery, and at 7 days, 6-8 weeks and 12-24 months post-partum. Samples were collected from the infants at birth and at 6-8 weeks, 14-16 weeks and 12 months of age.

### **Detection of NVPR mutations using the ViroSeq assay**

Plasma samples were analyzed with the FDA-cleared ViroSeq HIV-1 Genotyping System, which is based on population (bulk) sequencing of amplified DNA (see Methods). All of the samples collected from women prior to NVP or at delivery were wild type (no NVPR mutations detected, Figures 1a and 2a). Genotyping results were obtained for five of the nine women 7 days after NVP (one woman had no sample, and three women had samples with viral loads < 2,000 copies/ml that failed to amplify adequately for population sequencing). NVPR mutations were detected in samples from two of the five women: one had Y181C and one had Y181C+G190A. At 6-8 weeks after NVP, five women had K103N, three had K103N+Y181C,

and one had V108I. All of the samples collected 12-24 months after NVP were wild type.

All of the samples collected from infants at birth were wild type (Figures 1b and 2a). At 6-8 weeks after NVP, all five infants had NVPR mutations detected: two had Y181C, one had K103N, one had K103N+Y181C, and one had Y181C+Y188C. Samples collected 14-16 weeks after NVP were available for four of the five infants; one had Y181C and one had K103N. The G190A mutation was detected in one infant at 12 months of age; that mutation was not detected in samples collected from the same infant at earlier study visits. The 12-month samples from the other four infants were wild type.

All of the NVPR mutations detected in the samples from women and infants were detected as mixtures with wild type viruses, with the exception of the K103N mutation in the 6-8 week and 14 week samples from infant I-545. When K103N was detected, it was exclusively or predominantly encoded by the codon, AAC. The alternative codon, AAT, was present at a low level along with AAC in some samples. One exception was the 6-8 week sample from infant I-750, in which K103N was encoded by exclusively by AAT.

### **Optimization of the LigAmp assay for analysis of the K103N mutation in HIV-1 subtypes A and D**

HIV-1 genotyping assays based on population sequencing are designed to detect drug resistance mutations in the major viral population and are relatively insensitive for the detection of mutations that are present at low levels. Furthermore, those assays can not be used to quantify the level of resistant variants in the viral population. We used a novel assay, LigAmp [18], to detect and quantify HIV-1 variants with the K103N NVPR mutation. The K103N mutation was studied since it was the most common NVPR mutation selected in women in HIVNET 012 after

NVP exposure [3] and the most common mutation detected 6 months after NVP exposure in a South African cohort [12].

LigAmp is a two-step assay. The first step of the assay involves hybridization of two adjacent oligonucleotides to a DNA template. The 3' terminal nucleotide of the upstream ligation oligonucleotide is designed to hybridize (match) the template only if the template contains the mutation of interest. Hybridization of the 3' end of the upstream oligonucleotide to the template DNA is required for ligation of the two oligonucleotides to one another. The ligation oligonucleotides contain M13 tails that serve as primer binding sites in a subsequent, universal real-time PCR detection step. The upstream oligonucleotide also contains a foreign sequence (e.g. *LacZ*), which serves as a binding site for the Taqman probe in the real-time PCR detection step. In real-time PCR, a fluorescent dye is released from the probe during amplification, which allows the amplification to be monitored. A level of fluorescence is defined (threshold); the amplification cycle at which the threshold is achieved (cycle threshold) is inversely proportional to the amount of DNA in the initial reaction.

We optimized the LigAmp assay for detection and quantification of the K103N mutation (codon AAC at position 103 in HIV-1 RT) in subtype A and D HIV-1. First, we designed ligation oligonucleotides for each subtype, based on Ugandan consensus sequences (Table 1 and Figure 3). Sequences from Ugandan women and infants typically differ from one another at positions near the K103N mutation, reflecting the natural genetic diversity of HIV-1. To render the LigAmp assay less sensitive to sequence differences near K103N, the length of the ligation oligonucleotides was extended and the temperature of the ligation reaction was reduced (see Methods). Primers and probes used in the real-time PCR detection step of the LigAmp assay are universal and did not require any modification for detection of K103N in these subtypes.



To generate reference reagents, we isolated plasmids containing the HIV-1 *pol* region from Ugandan women with HIV-1 subtype A and D. The plasmids were selected to represent the genetic diversity typically observed among individuals of each subtype (Figure 3). Plasmids with and without the K103N mutation were isolated from each woman. The *pol* region of the plasmids was then re-amplified and mixtures of mutant and wild-type DNA were prepared for each woman at mutant DNA concentrations of 100%, 10%, 1%, 0.1%, 0.01% and 0%. The DNA dilution panels were then analyzed in the LigAmp assay using the subtype A- or D-specific ligation oligonucleotides. The standard curves generated for each dilution panel (% K103N vs. cycle threshold) were nearly identical (Figures 4a and 4b), despite the fact that the HIV-1 sequences from the women varied at the ligation oligonucleotide binding regions (Figure 3).

#### **Detection and quantification of the K103N mutation in plasma HIV-1 from women and infants using the LigAmp assay**

For the analysis of plasma HIV-1, we used PCR products produced in the ViroSeq system. PCR amplification in the ViroSeq system usually incorporates dUTP into the PCR products as part of a contamination control system. The presence of dUTP in the PCR products does not interfere with the LigAmp assay, but does interfere with cloning-based assays, such as the yeast TyHRT system (see below). Plasma samples available for this study were limited in volume. To permit analysis of the same samples with the yeast assay, samples were amplified without dUTP. PCR products from each sample were analyzed using LigAmp in three independent experiments. For each experiment, a standard curve (e.g. control dilution curve derived from one woman with the same HIV-1 subtype) was included as an internal control and was used to quantify the percentage of K103N in each sample (e.g. Figures 4c and 4d).

Results from the analysis of the maternal samples are shown in Figures 1a and 2a. In most cases, the % K103N in samples collected prior to NVP exposure or at the time of delivery was less than 0.1%. Selection of the K103N mutation was evident in eight of the nine women at 7 days and/or 6-8 weeks after NVP. In the 6-8 week samples, the mean % K103N among the eight women was 13.9%. In six women, the K103N mutation faded below 0.1% by 12-24 months after delivery (Figure 1a). However, in three women, K103N was detected above the pre-NVP level (at 0.8%, 1.3%, and 3.5%) a year or more after NVP administration (Figure 2a).

Results from the analysis of the infant samples are shown in Figures 1b and 2a. The % K103N was less than 0.1% in two of the infants at birth. In the other three infants, K103N was detected at a low level in at least one of the triplicate runs. The K103N mutation was detected above 0.1% in four of the five infants 6-8 weeks after NVP. The level of K103N remained high in one infant at the 14-16 week visit. In two infants, the mutation faded below 0.1% by 14-16 weeks. The fourth infant who had K103N detected at 6-8 weeks did not have a 14-16 week sample. In the infant who had high levels of K103N at the 6-8 and 14-16 week visits (I-545), K103N was detected at a level of 1.5% a year after single dose NVP (Figure 2a).

### **Comparison of results from the ViroSeq and LigAmp assays**

The % K103N detected by the LigAmp assay (mean of three experiments) was above 0.1% in 20 of the 63 samples tested. The ViroSeq assay detected the K103N mutation in 11 of those samples, including all samples where the mean LigAmp result was above 5%, and three samples where the % K103N was below 5% (4.2%, 3.7%, and 0.15%). In the sample with 0.15% K103N (the 6-8 week sample from infant I-750), the K103N mutation detected by ViroSeq was encoded by AAT, not by AAC, which was probed in the LigAmp assay.

### Analysis of NVPR using the TyHRT assay

Results from the LigAmp assay reveal that the K103N mutation persisted above the pre-NVP level in three women and one infant a year or more after single dose NVP (Figure 2a). We analyzed the samples from those women and infants using an independent method, the yeast TyHRT system, to confirm persistence of the K103N mutation. Samples from infant I-750, who had the G190A mutation detected in the 12-month sample using the ViroSeq assay, were also analyzed with the TyHRT assay for confirmation.

The TyHRT assay is a genetic assay that allows one to screen libraries of HIV-1 RT-containing clones for RT activity and NVP susceptibility. First, amplified HIV-1 *pol* region DNA from test samples is co-transformed into the yeast, *S. cerevisiae*, with a plasmid containing a TyHRT element with a deletion in the RT region. The HIV-1 RT DNA is introduced into the TyHRT element by homologous recombination. Each isolate carries a unique RT domain and the library of isolates is representative of the RT domains present in the original viral sample. The TyHRT elements carry the reverse transcription indicator gene, *his3AI*. Expression and reverse transcription of the TyHRT element by the recombined HIV-1 RT results in conversion of the *his3AI* gene into a functional *HIS3* gene [19], which enables the transformed yeast to grow on media lacking histidine. RT activity is selected for on media lacking histidine. Selection in the presence of NNRTIs measures the NNRTI susceptibility of individual RT clones. Analysis of the RT activity and NNRTI susceptibility [20] of the isolates present in large libraries makes it possible to detect NNRTI-resistant RT variants that are present at low frequency. Plasmids from selected NNRTI-resistant clones are sequenced to identify NNRTI resistance mutations in the HIV-1 RT coding region.

### **Comparison of results from the LigAmp and TyHRT assays**

Results from the LigAmp assay (% K103N) and the TyHRT assay (% NVPR) are shown in Figure 2b. Results from the two assays were consistent. Clones with phenotypic NVPR were isolated in the TyHRT assay from all of the samples that had K103N detected by the LigAmp assay. K103N was detected among the resistant clones in all but one of those samples (the 7 day sample from woman M-750). That sample had K103N detected at a low level in the LigAmp assay (mean = 0.06%); neither of the two NVPR clones that were isolated and sequenced in the TyHRT assay had the NVPR mutation. In three samples, phenotypic NVPR was detected in the TyHRT assay, but K103N was not detected in the LigAmp assay (the delivery sample from woman M-750, and the delivery and 7 day samples from woman M-474). NVP-resistant clones from those samples (2-8 clones per sample) isolated in the TyHRT assay did not have the K103N mutation. In the four long-term follow-up samples, K103N was detected by the LigAmp assay in the long-term follow-up samples at 1.3%, 3.5%, 0.8%, and 1.5% (for women M-750, M-830, and M-474 and infant I-545 respectively). The % NVPR detected in the TyHRT assay was 0.6%, 11%, 3.4%, and 0.3% for those samples, reflecting isolation of 2, 33, 7, and 1 NVP-resistant clones. Even though relatively few NVP-resistant clones were isolated, K103N was detected among the NVP-resistant clones from all four samples. In contrast, long-term follow-up samples from women and infants who did not have K103N detected by the LigAmp assay (Figure 1), had relatively few NVP-resistant clones isolated in the TyHRT assay, and none of the clones that were sequenced had K103N (data not shown). In the infant, I-750, the G190A mutation was detected by the ViroSeq assay in the 12-month sample. That mutation was also detected among NVP-resistant clones isolated from that sample in the TyHRT assay.

## **DISCUSSION**

Analysis of plasma HIV-1 using the LigAmp assay reveals persistence of the K103N mutation above pre-NVP levels in 3/9 women and 1/5 infants a year or more after single dose NVP administration. Presence of HIV-1 variants with the K103N mutation in the long-term follow-up samples from those individuals was confirmed using a second, independent method, the yeast TyHRT system. The ability of HIV-1 variants with K103N to persist for a year or more after single dose NVP exposure is consistent with data from *in vitro* studies that demonstrate that the K103N mutation confers a relatively small fitness cost [21-23]. Variants with the K103N mutation have also been shown to persist for years in the absence of antiretroviral exposure in some patients who are infected with resistant strains [24]. While K103N was the major mutation detected by the ViroSeq assay in women in HIVNET 012 after single dose NVP [3], Y181C was the most common mutation detected in infants [4]. This is consistent with our finding of lower levels of K103N in the infant samples using the LigAmp assay. Ligation oligonucleotides specific for Y181C and other NVPR mutations could be designed for the LigAmp assay to examine the emergence and fading of mutations other than K103N. Detection of G190A in one infant at 12 months of age using both the ViroSeq and TyHRT assays confirms that variants with other NVPR mutations can persist in infants for a year after single dose NVP. The length of time individual NVPR mutations persist in women and infants after single dose NVP is likely to depend on the impact of each mutation on viral replication once the drug is cleared. In HIVNET 012, HIV-1 subtype influenced the rate of fading of NVPR mutations in women between 7 days and 6-8 weeks after NVP administration [25]. Further studies are needed to evaluate the impact of HIV-1 subtype and other factors, such as viral load, on the long-term persistence of K103N

and other NVPR mutations after single dose NVP or other NVP-containing regimens used for pMTCT.

The LigAmp assay was particularly useful for quantification of K103N-containing variants in this study. This assay has an impressive linear range and can quantify variants down to a level of 0.1%. Greater levels of sensitivity can be achieved for detection of mutations in other systems (e.g. detection of cancer-associated mutations in genomic DNA) where genetic diversity around the mutation site is not an issue [18]. The assay can be performed without the need for patient-specific primers (which may be required for some mutation-specific real-time PCR assays for HIV-1 drug resistance mutations), and can be performed using PCR products remaining from routine genotyping. This was an advantage in this study, since the original plasma samples collected from women and infants were limited in volume. The assay is relatively simple to perform and uses very low concentrations of template, allowing one to perform replicate reactions for numerous drug resistance mutations from a single sample. Our previous report demonstrates that LigAmp can be used for simultaneous detection of different mutations [18]. A multiplex version of the LigAmp assay that could be used for simultaneous detection of different HIV-1 drug resistance mutations is in development.

The TyHRT assay also offers unique advantages for analysis of HIV-1 drug resistance mutations. That assay provides phenotypic selection of drug resistant variants, which can be further characterized by DNA sequencing. That approach may be particularly useful for analysis of low-level drug-resistant variants in non-B HIV-1 subtypes, where the genetic correlates of antiretroviral drug resistance are not well-defined.

In this report, the % K103N in long-term follow-up samples ranged from 0.8 to 3.5% (mean = 1.8%). Our preliminary analysis of HIVNET 012 samples with the TyHRT assay

suggests that other mutations, such as G190A, may also persist in some individuals after single dose NVP. Further studies are needed to determine whether persistence of NVPR mutations at low levels after single dose NVP prophylaxis compromises efficacy of NNRTI-containing regimens for HIV-1 treatment or pMTCT in subsequent pregnancies, especially years after NVP exposure. Emergence of NVPR in women and infants receiving regimens for pMTCT can usually be prevented by providing pregnant women with potent combination antiretroviral therapy. However, in resource-poor countries, there is often little access to antiretroviral drugs. Simpler regimens, such as the HIVNET 012 regimen, are more likely to be used for pMTCT. Some studies have evaluated the rate of NVPR in women who received single dose NVP for pMTCT in combination with other antiretroviral drugs. Using routine genotyping assays, NVPR was still observed in women after single dose NVP in PACTG 316 despite concurrent treatment with at least one active antiretroviral drug [6]. When single dose NVP was combined with short course zidovudine (ZDV) in the setting of pMTCT, NVPR was still seen in 21/74 (33%) of women in a study from the Ivory Coast [8], and in 33/190 (17%) of women from Thailand [26]. However, preliminary data from a clinical trial in South Africa suggest that addition of four or seven days of combivir (ZDV+3TC) to a single dose NVP regimen may reduce NVPR rates [27]. Further studies are needed to confirm those findings, and to evaluate the rate of NVPR in infants receiving single dose NVP in combination with ZDV or other drugs. Sensitive resistance assays, such as LigAmp, may be useful for evaluation of the persistence of resistant variants.

The HIVNET 012 regimen is safe, simple, and effective, and offers hope for preventing HIV-1 infection in thousands of infants around the world. If NNRTI-based regimens are found to be less effective for HIV-1 treatment or pMTCT in women and infants with prior single dose NVP exposure, and if the availability of antiretroviral drugs improves in resource-poor settings,

those women and infants could still be offered regimens for HIV-1 treatment and pMTCT that contain other antiretroviral drugs.

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## **TABLES**

**Table 1. Oligonucleotides and primers used in the LigAmp assay\***

### **Ligation Oligonucleotides**

ST	Position	Oligonucleotide sequence
A	Upstream	5' <u>ACTGTAAAACGACGGCCAGTGT</u> <u>CCCCCTCAA</u> <u>ACTGGCAGATGCAC</u> GAGGAATACCACATCCAGCAGGTCTAAAAAAGGAC-3'
	Downstream	5'AAATCAGTAACAGTACTAGATGTGGGGGTGGTCATAGCTGTTCC <u>TGCA</u> 3'
D	Upstream	5' <u>ACTGTAAAACGACGGCCAGTGT</u> <u>CCCCCTCAA</u> <u>ACTGGCAGATGCAC</u> GAGGAATACCACATCCTGCAGGGCTAAAAAAGGAC3'
	Downstream	5'AAATCAGTAACAGTACTGGATGTGGGTGTGGTCATAGCTGTTCC <u>TGCA</u> 3'

### **Real-Time PCR Primers and Probe**

M13 forward primer	5'-CTGTAAAACGACGGCCAGTG-3'
M13 reverse primer	5'-TGCAGGAAACAGCTATGACCA-3'
<i>LacZ</i> probe	FAM-5'-TCCCCTCAAACCTGGCAGATGCACG-3'-BHQ-1

\*The sequences of ligation oligonucleotides designed for detection of the K103N mutation (AAA→AAC) for subtype (ST) A and D are shown. Upstream and downstream ligation oligonucleotides include M13 tails (underlined). Upstream oligonucleotides also include a *LacZ* probe binding site (italics), and an intentional mismatch at the third position from the 3' end introduced to enhance the specificity of the LigAmp assay (G, bold). The sequences of primers and probes used in the real-time PCR detection step of the LigAmp assay are shown. The TaqMan probe includes a fluorophore (FAM) and a quencher (BHQ).

## **FIGURE LEGENDS**

**Figure 1. Analysis of HIV-1 from women and infants who did not have the K103N NVPR mutation detected in long-term follow-up samples.**

Plasma samples from women were collected prior to NVP (Pre), at delivery (Del), and at 7 days (7d), 6-8 weeks (6w) and 12, 14 or 24 months (12m, 14m, 24m) after delivery. Plasma samples from infants were collected at birth (B), and at 6-8 weeks (6w), 14 weeks (14w) and 12 months (12m) after delivery. Results from six women (M-847, M-790, M-660, M-470, M-554, M-755, Panel A) and four infants (I-466, I-827, I-750, I-788, Panel B) are shown. NVPR mutations detected using the ViroSeq system are shown below each graph. A dash (-) indicates that no NVPR mutations were detected. NR indicates that no result was obtained with the ViroSeq system. NA indicates that no sample was available for testing. Numbers indicate the positions of NVPR mutations (103=K103N, 108=V108I, 181=Y181C, 188=Y188C, 190=G190A). Polymorphisms at codons 135 and 283 were noted in some of the subjects (I135V in I-827; I135T in M-660, M-755, M-847, and I-788; L283I in M-755, I-788, and I-827). The percentage of variants with the K103N mutation was determined in triplicate for each sample using the LigAmp assay (% K103N). The mean and standard deviation from the three experiments are shown.

**Figure 2. Analysis of HIV-1 from women and infants who had the K103N NVPR mutation detected a year or more after single dose NVP administration.**

Panel A: Plasma samples from three women and one infant were analyzed using the ViroSeq and LigAmp assays as described in Figure 1. The I135V polymorphism was noted in samples from M-474 and M-750. Panel B: The same samples were analyzed using the TyHRT system (see

text). The percentage of colonies (HIV-1 variants) with NVPR is plotted for each sample (% NVPR). The table below the graph provides more detailed information, including the number of transformed clones (yeast colonies) isolated with active HIV-1 RT (# clones), the number of clones with NVPR (# NVPR), the number of clones with NVPR that were sequenced (# Seq), and the number of clones identified with specific NVPR mutations. In addition to the NVPR mutations listed in the Methods section, the following amino acid substitutions were noted: M-750: some clones had I135V; M-474: some clones had I135V, I132M, and M230I. I-545: some clones had V179I. Genetic linkage of NVPR mutations was observed in some clones. M-750 had one clone with V106A+Y188C (7 day sample), one clone with K103N+Y188C (6-8 week sample), and one clone with V106A+G190A (6-8 week sample). M-830 had two clones with K103N+Y181C (6-8 week sample).

### Figure 3. HIV-1 sequences from Ugandan women and infants

The sequence alignments for subtype A (panel A) and subtype D (panel B) include sequences of the HIV-1 binding regions of ligation oligonucleotides used for detection of the K103N mutation in the LigAmp assay (Oligos, see Table 1), sequences of the control plasmids used for analysis of subtype A (667, 842, 847, 687 and 703) and D (868, 638, 607), and sequences obtained using the ViroSeq system (population sequences) from each women (M) and infant (I) 6-8 weeks after single dose NVP. Consensus sequences are shown above each alignment. Nucleotides at the third position of codon 103 (bracket) are boxed. An A→G substitution at the first base of codon 103 in the upstream oligonucleotide (underlined) enhances specificity of the ligation reaction. Dots indicate nucleotides that match the consensus sequence. Nucleotide mixtures are indicated using IUB codes: M=A+C, R=A+G, Y=C+T, H=A+C+T, W=A+T.

Figure 4. Analysis of control plasmids and a representative plasma sample using the LigAmp assay.

Standard curves for detection of K103N (see below) were generated using reference plasmids from five women with subtype A (Panel A) and three women with subtype D (Panel B). Each curve was generated by testing serial dilutions of mutant DNA (with the AAC mutation for K103N) in the LigAmp assay. The % K103N in each control sample was plotted against the cycle threshold (Ct). Results for the control with no K103N (0% K103N) were similar for those obtained with the 0.01% K103N samples. In some experiments, either the 0% control sample or the 0.01% control sample failed to achieve threshold. Panels C and D show representative results from one experiment (analysis of the K103N mutation in woman M-750). The plot in Panel C shows fluorescent detection of the K103N mutation in the real-time PCR detection step of the LigAmp assay. The cycle threshold is indicated (horizontal line). Results from the analysis of an internal standard curve (10-fold dilutions of DNA containing the K103N mutation) are shown in blue (Std. 0.01-100). None of the three negative controls included in the experiment achieved cycle threshold (the 0% K103N control sample, the ligation control (no template), and the real-time PCR control (no template), data not shown). Results from analysis of samples from one woman (M-750) are shown in red (Pre: pre-NVP; Del: delivery; 7d: 7 days after single dose NVP; 6w: 6 weeks after single dose NVP, 14 mo: 14 months after single dose NVP). Panel D shows results from panel C plotted as the percentage of HIV-1 variants with the K103N mutation (% K103N) vs. cycle threshold. The standard curve (Std. curve, blue) was used to determine the % K103N in each plasma sample. Note that the highest % K103N was seen in the 6-week visit (open diamond), but that the % K103N was also elevated above baseline 14

months after single dose NVP (open triangle). The experiment shown in Panels C and D was performed in triplicate, and results from those experiments were included in the data presented in Figure 2.

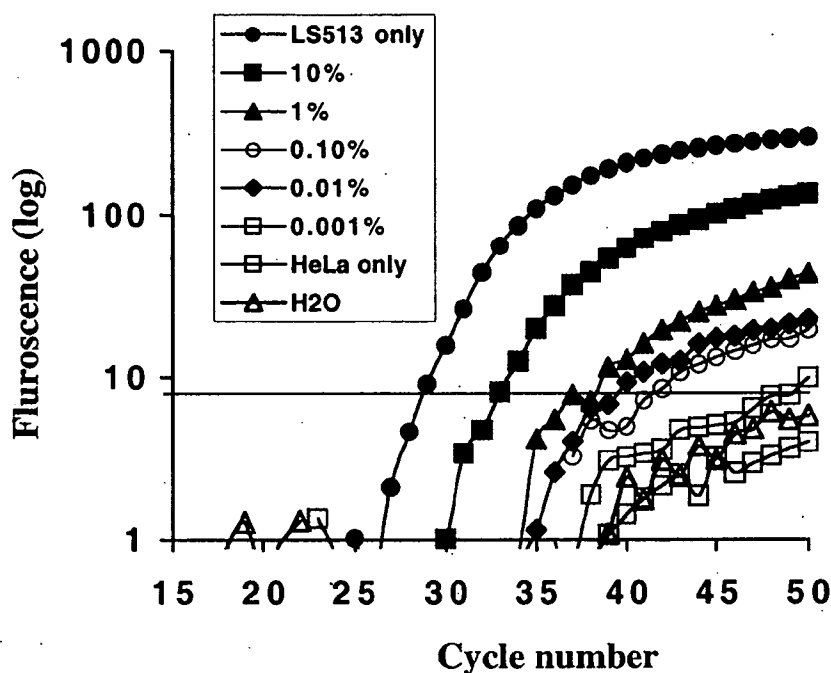


**We have expanded the LigAmp reaction in the following ways:**

- 1) Demonstrated that LigAmp quantitatively detects DNA from cell mixtures (in addition to DNA mixes previously demonstrated).
- 2) LigAmp on whole genome amplified DNA.
- 3) Gap LigAmp further increases specificity of the reaction.
- 4) Pancreas Cancer vs. Pancreatitis.
- 5) Detection of Methylated DNA using LigAmp.
- 6) LigAmp multiplexing using Bead detection of LigAmp products.
- 7) Bile duct Cancer early detection, bile and serum.
- 8) Braf mutation detection.
- 9) Mitochondrial mutations.
- 10) AACR Abstract: Shi C, Hua L, Parker AR, Wendelburg BJ, and Eshleman JR. LigAmp: Sensitive Detection of Single Nucleotide Differences. American Association for Cancer Research (AACR), Proceedings of the American Association for Cancer Research, 45: 986, 2004.
- 11) AMP abstract, submitted:  
Shi C, Fukushima N, Hua L, Parker AR, Wendelburg BJ, Yeo CJ, Hruban RH, Goggins MG, Eshleman JR. LigAmp: Sensitive detection of single nucleotide differences. Submitted AMP, 2004.
- 12) USCAP abstract, submitted:  
Ultrasensitive detection of kras mutations in bile and serum from patients with biliary cancer using ligamp technology. A Chandrasekharan, C Shi, P J Thuluvath, I Wistuba, C A Karikari, P Argani, M G Goggins, J R Eshleman and A Maitra
- 13) Final Nature Methods, manuscript in Press.  
Shi C, Eshleman SH, Jones D, Fukushima N, Hua L, Parker AR, Yeo CJ, Hruban RH, Goggins MG, and **Eshleman JR**. LigAmp: Sensitive Detection of Single Nucleotide Differences. Nature Methods, In Press.
- 14) Manuscript by Flys et al:  
T. Flys, DV. Nissley, CW. Claasen, D Jones, C Shi, LA Guay, P Musoke, F Mmiro, JN. Strathern, JB Jackson, JR. Eshleman, SH. Eshleman. "Sensitive drug resistance assays reveal long-term persistence of HIV-1 variants with the K103N nevirapine (NVP) resistance mutation in some women and infants after single dose NVP: HIVNET 012."

### 1) LigAmp on genomic DNA extracted from cell mixtures

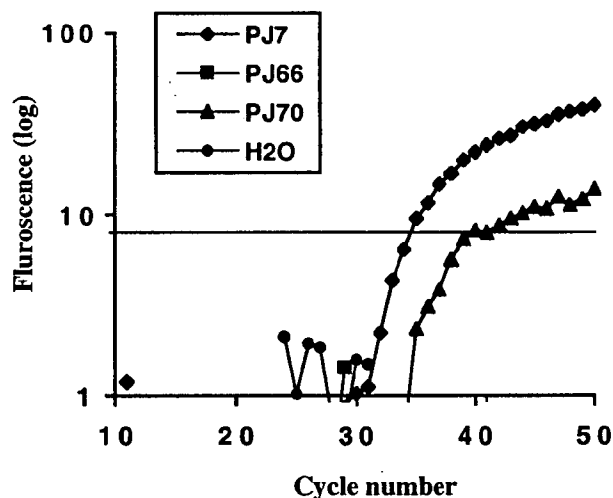
We have previously demonstrated that LigAmp is able to detect one mutant molecule in the present of 10,000 wild-type DNA copies using DNA mixtures. Here, we determined its sensitivity using genomic DNA isolated from previously made mixtures of mutant and wild-type cells. *KRAS2* mutant LS513 cells were serially diluted into wild-type HeLa cells. The percentage of the mutant cells in the cell mixtures ranged from 100% to 0.001%. One microgram of genomic DNA extracted from each cell mixture was used for LigAmp. As shown in the figure, using DNA isolated from cell mixture, the assay is able to detect one mutant cell in the presence of 10,000 to 100,000 normal cells.



## 2) LigAmp on whole genome amplified (WGA) DNA

Clinical samples such as serum, plasma and other body fluids often contain only small amounts of DNA, which makes it difficult to detect mutant DNA directly using DNA isolated from these samples. Moreover, PCR amplification is commonly used to exponentially produce many copies of a DNA target sequence, but restricts one to a single gene or at most a handful of genes of interest. PCR based whole genome amplification is notoriously biased (not even amplification). Since LigAmp has the potential to multiplex many different point mutations, we therefore wished to demonstrate whether LigAmp could be performed on WGA DNA.

We therefore performed LigAmp on whole genome amplified DNA. One nanogram of DNA from pancreatic cancer patient's pancreas juice samples PJ7 (GAT), PJ66 (GGT) and PJ70 (GAT) were subjected to whole genomic amplification. Two hundred nanograms of amplified products were then used as template for LigAmp detection of GAT mutant. Data shows that LigAmp was capable of detecting the KRAS2 mutation in whole genomic amplified DNA in PJ7 and PJ70, consistent with our previous data using PCR-amplified DNA (Shi et al, Nature Methods, 2004).

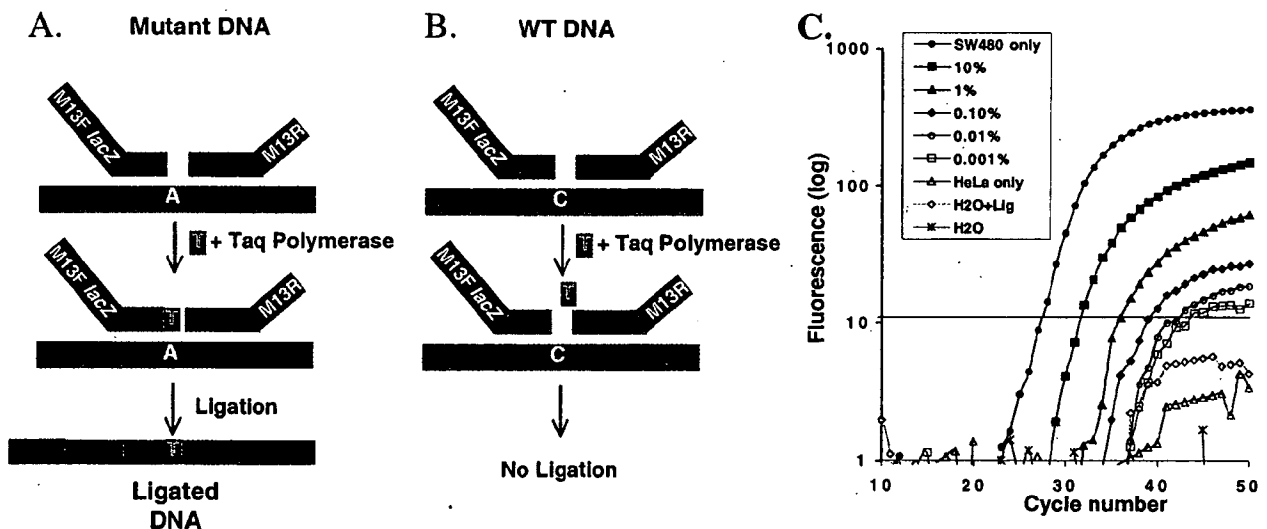


### 3) GAP LigAmp

LigAmp assay is a very sensitive point mutation detection strategy, able to detect one mutant DNA molecule in the presence of 10,000 wild-type copies. However, in initial work, the amplification signal derived from 1: 100,000 DNA mixtures overlapped with that from wild-type DNA only. The specificity of the assay is mainly determined by fidelity of the DNA ligase used in ligation step. Although we used a thermostable DNA Ligase (pfu DNA ligase) to enhance the specificity, misligation still occurs.

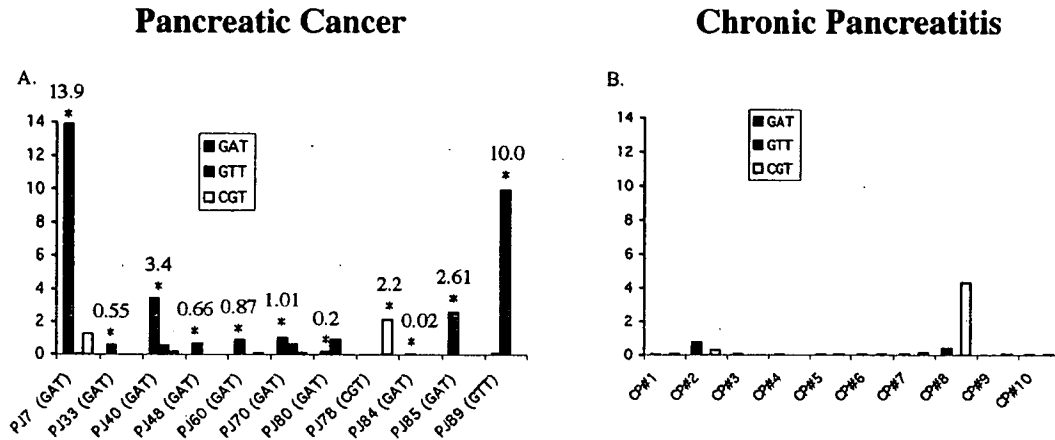
To further increase specificity, we included a Gap, similar to that included in Gap-LCR to improve specificity in that reaction. We call the modified reaction GAP-LigAmp. In standard LigAmp, upstream and downstream oligonucleotides bind adjacently to each other upon hybridization to the target. In GAP-LigAmp, after hybridization to the target, a gap of one or several bases is present between these two oligonucleotides. Taq DNA polymerase and the specific nucleotide corresponding to the mutant base were added into the ligation reaction to fill the gap, and the resultant oligonucleotides can be joined by DNA ligase (Figure A and B). When using wild-type DNA as a template, the corresponding wild-type base is not included in the reaction, therefore, no extension should occur. In the event that extension still does occur, no ligation should take place. There are then two steps that must both occur for ligation to occur and therefore two independent layers of specificity.

To determine the sensitivity of GAP-LigAmp, we serially diluted a KRAS2 mutant SW480 DNA (G12T) into wild-type HeLa DNA (GGT). The compositions in the ligation reaction are similar to that in LigAmp, except that GAP upstream oligonucleotide, Taq DNA polymerase (1 U) and dTTP (20 mmol). The extension/ligation condition are: 95C for 4 min followed by 99 cycles of 95 C for 30 seconds and 60 C for 4 mins. As shown in the panle C below, GAP-LigAmp is able detect mutant KRAS2 at a level of 0.001% (1: 100,000). Wild-type signal is undetectable (does not cross threshold). This result suggests that introduction of a GAP into the reaction further improves the specificity of LigAmp.



#### 4) LigAmp for early detection of Pancreas Cancer.

We have demonstrated that LigAmp can be applied to early detection of cancers. We have expanded the series of pancreas cancers patients tested. As shown below, the LigAmp assay detected the mutation present in the pancreas cancer. In all cases, this was the dominant mutation detected, except for one case. An expanded series of samples and comparison to additional controls is being performed.

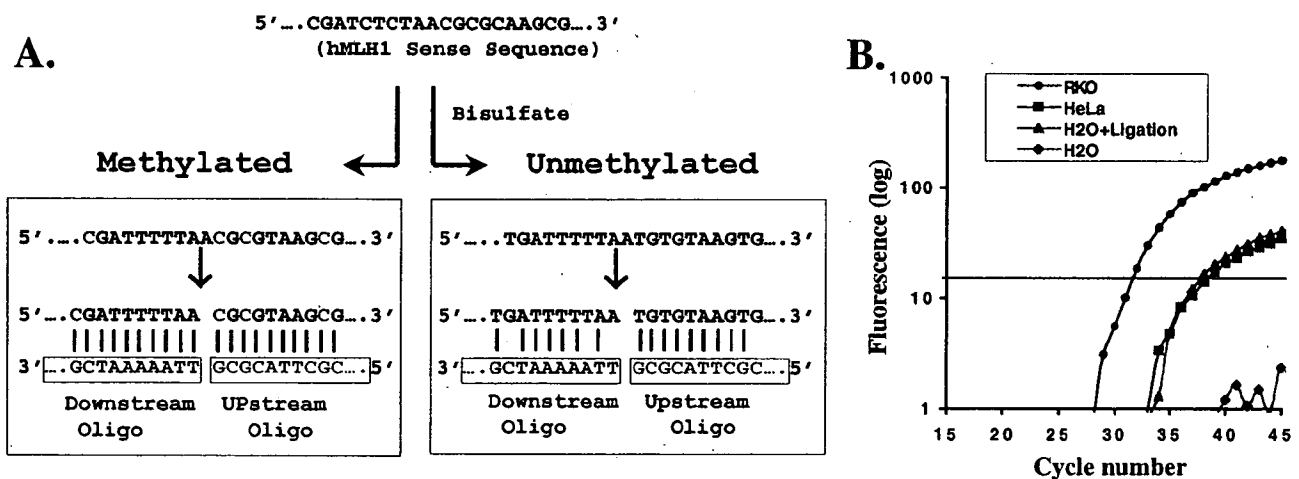


## 5) Application of LigAmp in detection of methylated DNA

DNA methylation has been used as a biomarker to distinguish tumors from normal tissues. The most widely used assay for detection of methylation levels is methylation-specific PCR (MS-PCR). In MS-PCR, unmethylated cytosines are first converted into thymine by sodium bisulfate treatment, while methylated cytosines in CpG islands are refractory to the treatment, thereby converting methylation information into sequence difference. The DNA is then amplified using primers designed specifically for methylated or unmethylated DNA. LigAmp was designed to detect point mutations. Single base differences between methylated and unmethylated DNA are created after bisulfate modification. Therefore, we hypothesized that LigAmp could also be applied to detect methylated DNA.

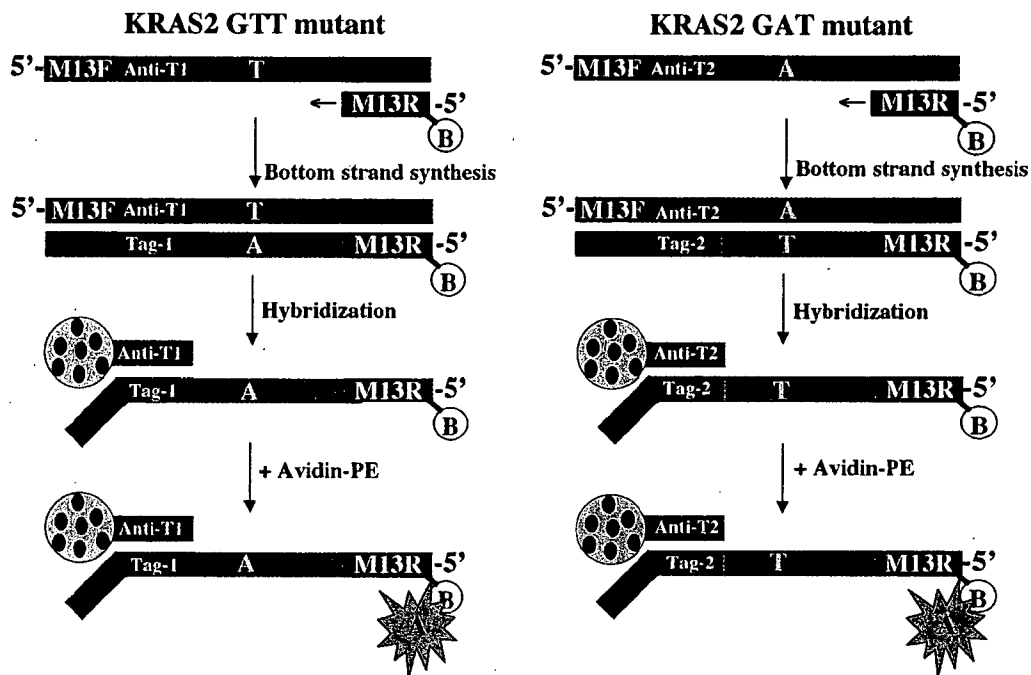
In colon cancer, methylation of *hMLH1* promoter region accounts for some MSI tumors. Analysis of *hMLH1* methylation might be helpful for colon cancer and other cancer detection. Here, we used *hMLH1* as a model to test our hypothesis. Methylation of a small proximal region (-248 to -178 relative to transcription start site) in the promoter are associated with loss of *hMLH1* expression. We selected a portion of this region as a target for ligation oligonucleotides. The oligonucleotides were designed to hybridize to the methylated sequence following bisulfate treatment. The 3'-end of the upstream oligonucleotide pairs with the cytosine base of a methylated CpG island (figure A). Multiple CpG islands in the upstream target site, might enhance the specificity, but are not required for the assay.

Genomic DNA was extracted from an *hMLH1* methylated colon cell line, RKO, and an *hMLH1* unmethylated cell line, HeLa (as a negative control). One microgram genomic DNA was first subjected to sodium bisulfate treatment and subsequently column purification. The modified DNA (200 ng) was used as ligation template. The Q-PCR Amplification signal for RKO appears earlier than HeLa (shown in the figure B). The difference in Ct values between RKO and HeLa is 7 cycles. The signal from HeLa DNA overlaps with that from H<sub>2</sub>O LigAmp control. This data demonstrate that LigAmp can be used to detect methylation.



## 6) Multiplex LigAmp with simultaneous Bead Detection.

We have demonstrated that the LigAmp reaction can be multiplexed using real-time PCR as the detection system. However realtime PCR can only be multiplexed to a certain degree before all of the fluorescent channels are exhausted (typically about 4 channels currently). We therefore wished to test whether LigAmp could be performed using bead detection. Using the Luminex system, 100 beads can be distinguished from each other using 10 different levels of 2 independent fluorophors. Each of these beads also contains a DNA tag (Flexmap system). Therefore instead of using LacZ or 16SrDNA as probes, one can substitute the tags present on the beads. Such a system is illustrated below. In this system, 2 different KRAS2 mutations are detected, each on one of the uniquely colored beads. A third bead can be used to detect wildtype KRAS. Other beads can be used to detect p53 mutations, Braf mutations, etc.



## **7) Bile duct cancer early detection-Bile and Serum analysis**

### **LigAmp of KRAS2 mutation on bile and serum collected from patients with biliary cancer**

Many patients with biliary tract carcinoma were diagnosed at an advanced stage, resulting a poor prognosis. Early detection of biliary cancer might improve the survival rate. Analysis of cancer-related genes present in bile or serum may provide a tool for early diagnosis. The genetic alterations such KRAS2 and p53 mutations were observed in sporadic biliary tract cancer. Here, we demonstrate that LigAmp can be applied in KRAS2 mutation detection in bile and serum samples from patients with biliary cancer.

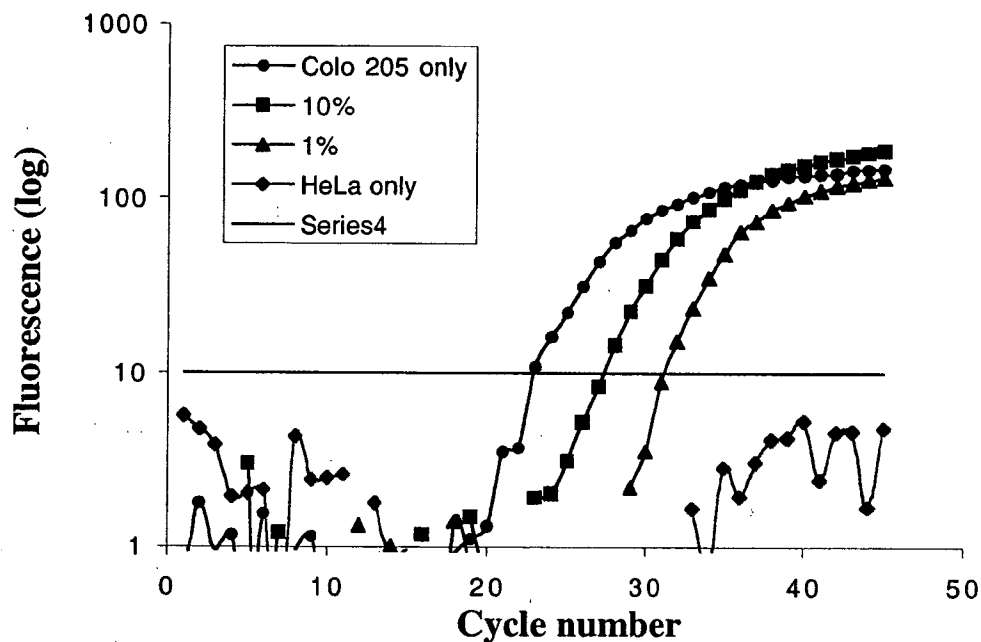
DNA isolated from cancer tissues, bile and serum were first subjected to PCR amplification. Five hundred picogram PCR DNA was used as template for LigAmp. LigAmp on DNA from the biliary cancer tissues demonstrated that majority of cancer (more than 90%) harbored GAT mutation. Mutation of KRAS2 (GAT) was detected in 81% (13/16) of bile from patient with biliary cancer, while 21% (6/28) of bile from control group. These six control patients with KRAS2 mutation carried diseases associated with chronic inflammation. In the serum DNA from biliary cancer patients, 54.5% (6/11) were detected to contain KRAS2 (GAT) mutant DNA. These data shows that LigAmp on bile and serum can be used an early diagnosis tool.



### 8) Braf mutation detection using LigAmp

Point mutations of the *BRAF* protooncogene were recently found in a wide variety of tumors, and most notably in melanoma, papillary thyroid cancer and colon cancer. A V599E (T1796A) hotspot mutation within the *BRAF* gene was reported in these tumors. Therefore, *BRAF* can be a promising tumor marker for these malignancies.

To demonstrate the capability of LigAmp to detect *BRAF* mutations, we serially diluted *BRAF* mutant genomic DNA (from 1.2  $\mu$ g to 12 ng) isolated from a colon cancer cell line, Colo205, and mixed with wild-type DNA (1.2  $\mu$ g) from HeLa cells. The 3'-terminal base of the upstream ligation oligonucleotide for the mutant was designed to perfectly pair with the mutated base as usual. The result shows that LigAmp is able to detect and quantify the *BRAF* mutation in the DNA mixtures.



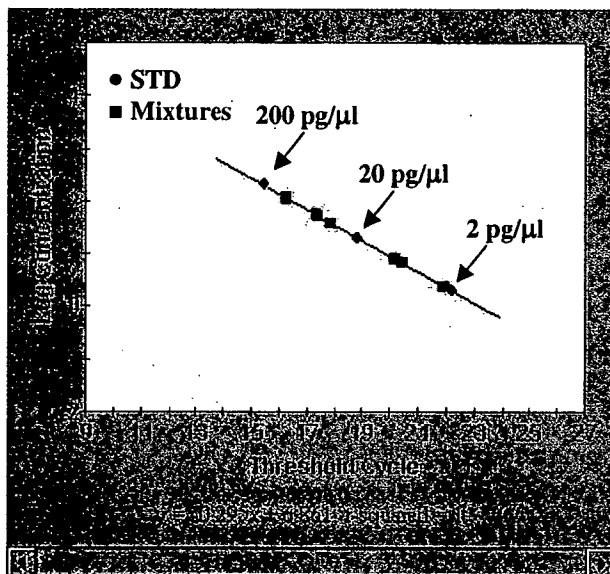
## 9) Mitochondrial mutation detection

### LigAmp to detect heteroplasmic mitochondria

Heteroplasmic mutations of mitochondrial DNA are an important source of human diseases. Detection of low levels of heteroplasmic is essential for both the diagnosis of some medical disorders, as well as for forensic identify determinations. Here, we wanted to test if LigAmp can be used to detect heteroplasmy using human mitochondrial genomic DNA.

The mutant (C6371T) mitochondrial DNA was diluted into the wild-type mitochondrial DNA ranging from 1% to 50%. Then, the mixtures were subjected to PCR amplification. Two hundred pg PCR product was used as template for LigAmp. The result is shown in the figure. The data demonstrate the ability of LigAmp to detect heteroplasmy in mitochondria at a low level.

A. Standard curve for quantification



B. % heteroplasmy in the mixtures

Sample #	Heteroplasmy (%)
A	55.3
B	18.7
C	3.3
D	55.7
E	1.1
F	27.5
G	4.02
H	25.6

**10) AACR Abstract: Shi C, Hua L, Parker AR, Wendelburg BJ, and Eshleman JR. LigAmp: Sensitive Detection of Single Nucleotide Differences. American Association for Cancer Research (AACR), Proceedings of the American Association for Cancer Research, 45: 986, 2004.**

### **LigAmp: Sensitive Detection of Single Nucleotide Differences**

Chanjuan Shi<sup>1</sup>, Li Hua<sup>1</sup>, Antony R. Parker<sup>1</sup>, Brian Wendelburg<sup>3</sup>, and James R. Eshleman<sup>1,2</sup>

Sensitive and accurate detection of small number of tumor cells in the presence of a vast excess of normal cells is a problem common to many cancer research and clinical applications, but it is difficult since the mutant DNA often differs from the wildtype DNA by only a single base. The aim of present study is to develop a novel strategy that converts a single base substitution to a distinct molecule so that it can be detected in a sensitive and linear fashion. This strategy (designated LigAmp) employs two unique oligonucleotides that contain regions specific to the target gene and M13 tails. In addition, the upstream oligonucleotide also contains a region of completely foreign DNA. These two oligonucleotides should be ligated at the mutation site only when a perfectly matched target is present. Ligated products are then amplified by realtime quantitative PCR using M13 primers and the foreign DNA region as a probe. To test this strategy, K-ras mutant SW480 genomic DNA was 10 fold serially diluted into wildtype K-ras Hela DNA. We demonstrated the ability to detect one mutant DNA molecule in the presence of a background of 10,000-100,000 wildtype molecules. We envision that with this level of sensitivity and accurate quantification, this approach may find many cancer applications, including early detection, minimal residual disease testing and molecular relapse monitoring.

**11) AMP abstract, submitted: Shi C, Fukushima N, Hua L, Parker AR, Wendelburg BJ, Yeo CJ, Hruban RH, Goggins MG, Eshleman JR. LigAmp: Sensitive detection of single nucleotide differences. Submitted AMP, 2004.**

**LigAmp: Sensitive Detection of Single Nucleotide Differences**

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Sensitive detection of small number of tumor cells in a vast excess of normal cells is a problem common to many cancer research and potential clinical applications. The mutant DNA often differs from the wild-type DNA by only a single base. We developed a novel strategy that converts a single base substitution into a completely foreign molecule. This strategy (LigAmp) employs two unique oligonucleotides that contain regions specific to the target gene and M13 tails. In addition, the upstream oligonucleotide contains a region of completely foreign DNA. These two oligonucleotides should be ligated at the mutation site only when a perfectly matched target is present. After conversion, ligated products are amplified by realtime quantitative PCR using M13 primers and a probe to the foreign DNA region on a Cepheid SmartCycler. To test this strategy, *KRAS2* mutant genomic DNA was 10-fold serially diluted into wildtype DNA. We demonstrate the ability to detect one mutant DNA molecule in the presence of a background of 10,000 wild-type molecules. LigAmp can be multiplexed, detecting different mutations, or both mutant and wild-type DNA, simultaneously. In a model of early detection, *KRAS2* mutations were detected in pancreatic duct juice from patients with pancreatic cancer. The relative amounts of *KRAS2* mutant to wild-type DNA were obtained by absolute quantification. LigAmp permits sensitive and linear detection of point mutation containing DNA. We envision that with this level of sensitivity and accurate quantification, this approach may find several cancer, infectious disease and genetics applications.

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**ULTRASENSITIVE DETECTION OF KRAS MUTATIONS IN BILE AND SERUM FROM PATIENTS WITH BILIARY CANCER USING LIGAMP TECHNOLOGY**

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**Background:** Biliary cancer is a lethal disease, and early detection efforts are needed to ameliorate the dismal prognosis. Mutations of the KRAS gene, specifically at codon 12, are one of the most common genetic aberrations in this cancer. An ultra-sensitive technology LigAmp - has been described (Shi et al, Nature Methods, 2004) for the detection of single base pair mutations in clinical samples. LigAmp has a sensitivity of detecting a mutant population with a sensitivity of 1:10,000 wild-type cells. We utilized LigAmp to detect KRASG12D mutations in patients with a variety of neoplastic and non-neoplastic biliary diseases.

**Design:** In LigAmp, a mutation specific 5 oligonucleotide and a generic 3 oligonucleotide (both tagged with M13 tails) are ligated using Pfu ligase, followed by amplification using M13 primers. The 5 oligonucleotide also has an upstream unrelated bacterial gene sequence (e.g., lacZ), and a specific flurophor-labeled probe to the latter can be utilized to generate cycle threshold (Ct) values for the mutant DNA of interest in the sample. Serially diluted positive control and negative control cell lines in each run provide relative quantification of mutant KRAS levels. Oligonucleotides specific to the KRASG12D mutation were designed. DNA was extracted from 119 samples, including 10 biliary cancer xenografts, 54 archival biliary cancers, 44 bile samples, and 11 serum samples. Of the 44 bile samples, 16 were from patients with biliary cancers, and 28 from a variety of non-neoplastic pancreato-biliary disorders; all 11 serum samples were from patients with biliary cancer.

**Results:** KRASG12D mutations were detected in 10/10 (100%) biliary xenografts and 52/54 (96%) archival cancers. 13/16 (81%) neoplastic bile samples and only 6/28 (21%) non-neoplastic bile samples harbored mutant KRAS DNA ( $P=0.0003$ ); the latter included chronic pancreatitis and primary sclerosing cholangitis, both conditions where this mutation has been reported. KRASG12D mutations were also detected in 6/11 (55%) serum samples from biliary cancer patients.

**Conclusion:** KRASG12D mutations are present in the majority of biliary cancers, and are detectable in bile and serum using LigAmp. This technology has the potential for early detection of biliary cancer as well as for disease monitoring post-therapy.

# LigAmp for sensitive detection of single-nucleotide differences

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**We developed the LigAmp assay for sensitive detection and accurate quantification of viruses and cells with single-base mutations. In LigAmp, two oligonucleotides are hybridized adjacently to a DNA template. One oligonucleotide matches the target sequence and contains a probe sequence. If the target sequence is present, the oligonucleotides are ligated together and detected using real-time PCR. LigAmp detected *KRAS2* mutant DNA at 0.01% in mixtures of different cell lines. *KRAS2* mutations were also detected in pancreatic duct juice from patients with pancreatic cancer. LigAmp detected the K103N HIV-1 drug resistance mutation at 0.01% in plasmid mixtures and at ~0.1% in DNA amplified from plasma HIV-1. Detection in both systems is linear over a broad dynamic range. Preliminary evidence indicates that reactions can be multiplexed. This assay may find applications in the diagnosis of genetic disorders and the management of patients with cancer and infectious diseases.**

Single-base mutations have an important role in cancer and other human diseases, and they can be useful markers for diagnostic tests. Mutations in viral genomes can cause resistance to antiviral drugs. Unfortunately, clinical samples often contain small numbers of mutant cells and viruses mixed with a vast excess of wild-type cells or viruses; thus the development of sensitive and accurate methods for detection of point mutations is an important challenge.

Single-base mutations can be detected with restriction-fragment-length polymorphism–Southern blot assays<sup>1,2</sup>, oligonucleotide ligation assays<sup>3,4</sup> and allele-specific PCR (AS-PCR) assays or the amplification-refractory mutation system<sup>5–8</sup>. The limit of detection of those assays is often confined to 0.1–1% (mutant/wild-type ratio of 1:1,000–1:100). Other assays can detect single-base mutations in unique sequence contexts at higher ratios<sup>9–12</sup> but cannot be generally applied. Detection of point mutations with real-time quantitative PCR (Q-PCR) assays is often hampered by cross-hybridization of probes to wild-type templates<sup>13</sup>.

New initiatives support development of assays for early diagnosis of common cancers in families at increased risk and also in population screening<sup>14–16</sup>. Such assays may also help in monitoring

bone marrow transplant engraftment<sup>13</sup> and disease recurrence in cancer patients after treatment. Cancer of the pancreas is usually diagnosed at an advanced stage, and early diagnosis is crucial for improving survival rates. *KRAS2* mutations are present in most cancers of the pancreas and can be detected in pancreatic duct juice as well as in plasma and stool<sup>17,18</sup>. However, *KRAS2* mutations can also be detected in pancreatic duct juice and stool from patients with chronic pancreatitis or pancreatic intraepithelial neoplasias (PanINs)<sup>19,20</sup>. Although the detection of mutant *KRAS2* alone is not an accurate predictor of pancreatic cancer<sup>21</sup>, quantitative assays for *KRAS2* mutations in biological fluids might be able to distinguish between pancreatic cancer and other conditions<sup>22</sup>.

Assays for sensitive detection of point mutations may also aid in management of patients with HIV-1 infection. Antiretroviral drugs can select for HIV-1 with drug resistance mutations in protease and reverse transcriptase. Most HIV-1 genotyping assays are relatively insensitive for detection of minority variants with resistance mutations. A recent study suggests that the presence of such variants can influence treatment response (Mellors, J. *et al.*, *Antiviral Ther.* 8, S150, 2003). A multicenter study used different assays to detect the K103N drug resistance mutation (Halvas, E. *et al.*, *Antiviral Ther.* 8, S102, 2003). Two AS-PCR assays and a yeast hybrid assay specific for HIV-1 reverse transcriptase detected the mutation at <1%. Other assays were less sensitive.

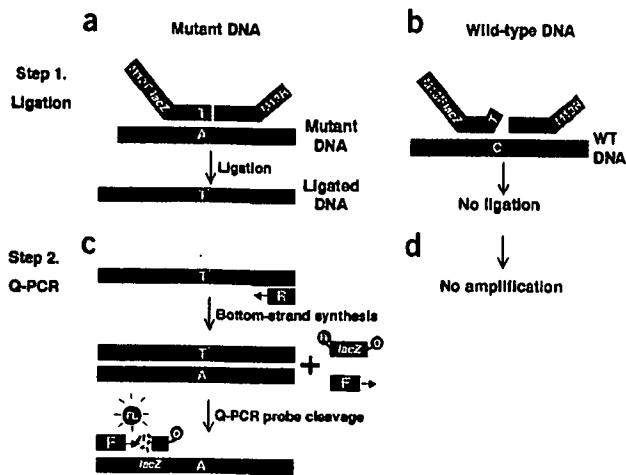
We report here a simple, universal strategy for sensitive detection and quantification of single-base differences. Two model systems were used for assay development: (i) detection of *KRAS2* and *TP53* mutations for early detection of cancer and (ii) detection of the K103N HIV-1 drug resistance mutation.

## RESULTS

### Overview of the LigAmp assay

We designed the LigAmp assay to convert single-base differences into more distinctive molecules that could be easily detected and quantified. In the first step, two oligonucleotides are hybridized to a DNA template and ligated to one another (Fig. 1a). Each contains a region specific to the target gene (green) and an M13 tail (blue). The M13 tails permit amplification of the ligated product in a subsequent universal Q-PCR detection reaction. The upstream

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**Figure 1** | Overview of the LigAmp assay. (a–d) The assay includes two steps: (a,b, step 1) template-dependent ligation of two oligonucleotides and (c,d, step 2) detection and quantification with Q-PCR. Details of the LigAmp assay are described in the Results section. F, Forward M13 primer; R, reverse M13 primer; FL, fluorophore; Q, quencher. The asterisk indicates the terminal thymidine base on the upstream mutant oligonucleotide.

oligonucleotide also contains a region of unique foreign DNA (for example, *lacZ* DNA, red) that serves as the binding region for a probe in the Q-PCR reaction. We designed the upstream oligonucleotide to match either the mutant or wild-type sequence at the 3' end. When an upstream mutant oligonucleotide is used (Fig. 1a), we designed the 3' end of the oligonucleotide to match the mutant template perfectly. The same oligonucleotide should mispair at the 3' end when hybridized to a wild-type template, preventing ligation (Fig. 1b).

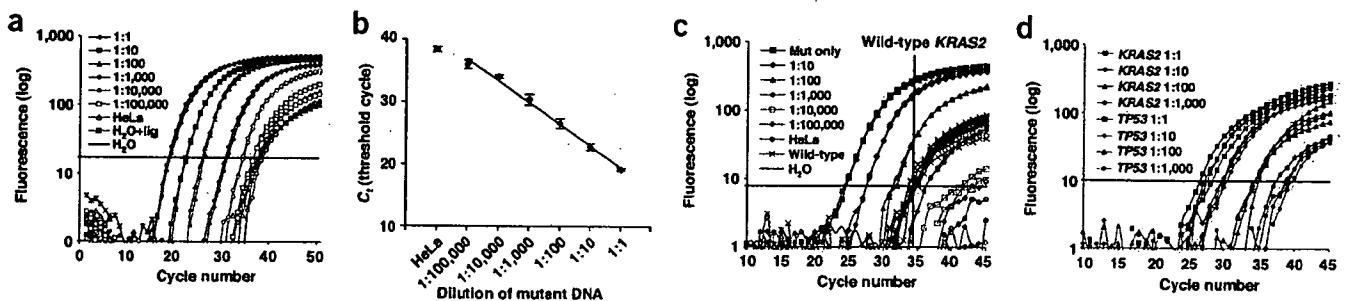
In the second step, we amplified the ligated DNA using M13 primers and detected it in a Q-PCR reaction (Fig. 1c). This step is independent of the specific gene or mutation targeted in the

ligation step. We detected Q-PCR amplicons using a universal probe (such as *lacZ*) that contains a fluorophore and quencher. Because both the M13 forward primer and the *lacZ* probe have the same polarity as the upstream ligation oligonucleotide, the *lacZ* probe cannot bind to the ligation oligonucleotide. Binding of the probe requires ligation of the two oligonucleotides (Fig. 1a) and subsequent polymerization of the complementary (bottom) strand of DNA in the Q-PCR step (Fig. 1c). The Q-PCR probe binds to the bottom strand of the amplified DNA. Extension from the M13 forward primer allows the probe to be cleaved and the fluorophore detected. If no ligation occurs in the first step because of mispairing (Fig. 1b), there is no template for amplification in the Q-PCR step (Fig. 1d).

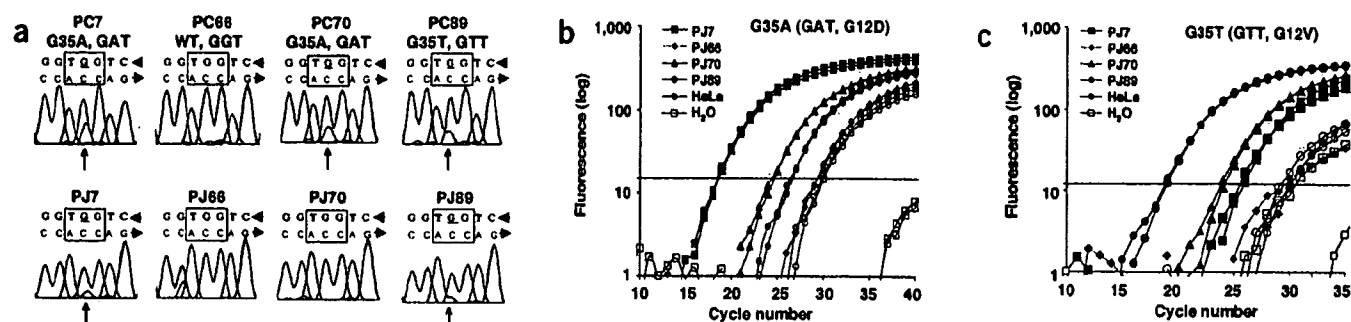
Because this strategy involves a ligation step followed by an amplification-detection step, we have designated it 'LigAmp'. We used LigAmp to detect and directly quantify mutant DNA alone (Fig. 1). Alternatively, we used a pair of mutant and wild-type upstream oligonucleotides to simultaneously detect and quantify mutant and wild-type DNA. In either case, the specificity of LigAmp relies on the differentiating power of a DNA ligase to ligate the upstream and downstream oligonucleotides only when both hybridize to the template with no mismatches at the adjacent terminal nucleotides.

#### Detection of the *KRAS2* mutation in cell line DNA mixtures

To demonstrate proof of principle, we serially diluted DNA from the SW480 colon cancer cell line, which contains a *KRAS2* mutation (G35T, GTT, G12V), with HeLa cell line DNA, which contains wild-type (GGT) *KRAS2* alleles. First we ligated the mutant-specific oligonucleotide to a common oligonucleotide, and then we conducted Q-PCR using M13 primers and a *lacZ* probe. We detected mutant *KRAS2* DNA as a signal distinct from HeLa DNA at a 1:10,000 dilution (Fig. 2a), and the signal was linear over a broad range of dilutions (Fig. 2b,  $r^2=0.99$ , least-squares analysis). Omission of ligase from the ligation reaction resulted in no amplification (data not shown).



**Figure 2** | Detection of genomic DNA containing the *KRAS2* mutation. In Q-PCR, amplification occurs at different cycle numbers depending on the initial template concentration. A fluorescence threshold is defined, and the cycle at which this threshold is crossed is determined for each sample (horizontal red line, Ct). (a) Representative Q-PCR amplification curves (in duplicate) of *KRAS2* mutant SW480 DNA serially diluted into wild-type (WT) *KRAS2* HeLa DNA. The 'H<sub>2</sub>O + lig' sample is a water control that was subjected to both the ligation and Q-PCR steps; the 'H<sub>2</sub>O' sample was subjected only to the Q-PCR step. (b) The mean Ct values (six independent assays) were plotted against the dilution of mutant DNA. Error bars represent 1 s.d. The mean Ct of the 1:10,000 mutant DNA sample plus 3 s.d. does not overlap with the mean Ct of the HeLa wild-type DNA minus 3 s.d. (c) Multiplex detection of mutant and wild-type *KRAS2* (in duplicate). *KRAS2* mutant SW480 DNA was diluted serially 10-fold into HeLa DNA, and the signals for mutant and wild-type *KRAS2* were simultaneously detected in Q-PCR using *lacZ* and 16S rDNA probes, respectively. The upstream *KRAS2* wild-type primer was used at a lower concentration (see Methods). The wild-type *KRAS2* signals for each sample are superimposed (overlapping red curves) with Ct of ~34 cycles (black vertical line). The other curves reflect detection of mutant DNA with the mutant probe. (d) Multiplex LigAmp for *KRAS2* and *p53*. Detection of the *p53* G818A mutation (CAT, R273H) in SW480 DNA (blue), and the *KRAS2* G35A mutation (GAT, G12D) in LS513 DNA (red) using multiplexed ligation oligonucleotides and the *lacZ* and 16S rDNA probes. Both DNA samples were serially diluted with HeLa-cell DNA.



**Figure 3 | Detection of mutant *KRAS2* sequences in pancreatic duct juice from pancreatic cancer patients.** (a) *KRAS2* DNA was amplified from samples of pancreatic cancer tissue (PC, top row) and corresponding samples of pancreatic duct juice obtained from the same patients at the time of surgery (PJ, bottom row), and sequenced as described (see Methods). The electropherograms show antisense sequence. Antisense sequence text (bottom strand, right arrowheads) is in a 5'-to-3' orientation, and the corresponding sense sequence text (top strand, left arrowheads) is in a 3'-to-5' orientation. The three bases for codon 12 are boxed. Peaks representing nucleotide mixtures at codon 12 are indicated below each electropherogram (arrows), and the mutant bases are underlined in the sense sequence. (b,c) LigAmp detection of the G35A (GAT, G12D) and G35T (GTT, G12V) mutations, respectively, in four pancreatic duct juice samples. HeLa DNA was included as a negative control.

### The LigAmp reaction can be multiplexed

We simultaneously detected mutant and wild-type *KRAS2* DNA in a multiplex reaction by including both mutant and wild-type oligonucleotides in one ligation reaction. To maintain a broad linear range of mutant DNA detection, we substantially reduced the concentration of the upstream wild-type primer (see Methods). We detected mutant DNA with a *lacZ* probe and wild-type DNA with a 16S rDNA probe containing a different fluorophore. When mutant SW480 DNA was serially diluted into wild-type HeLa DNA, the mutant DNA cycle threshold (Ct) varied with the concentration of input DNA, whereas the wild-type DNA Ct was relatively constant (Fig. 2c). We also used LigAmp to simultaneously detect *KRAS2* and *TP53* mutations in a single ligation reaction (Fig. 2d). The multiplexed reaction detected each mutation in either SW480 DNA or LS513 DNA at dilutions up to 1:1,000. Additional experiments are needed to determine the precise impact of multiplexing on the sensitivity of LigAmp reactions.

### Detection of tumor-specific *KRAS2* mutations in pancreatic juice

We next tested whether LigAmp could detect *KRAS2* mutations in samples from patients with pancreatic cancer. We first amplified and sequenced the hot-spot region of the *KRAS2* gene from four pancreatic cancer (PC) tissue samples after microdissection. Two samples had the G35A (GAT, G12D) mutation (PC7 and PC70), one had wild-type (GGT) *KRAS2* alleles (PC66) and one had the G35T (GTT, G12V) mutation (PC89) (Fig. 3a, top row, arrows). We then sequenced pancreatic duct juice (PJ) samples collected from the same patients at the time of surgery (Fig. 3a, bottom row). Two samples (PJ7 and PJ89) had small peaks in the sequencing electropherograms that corresponded to mutations detected in the tumor samples from the same patients (arrows).

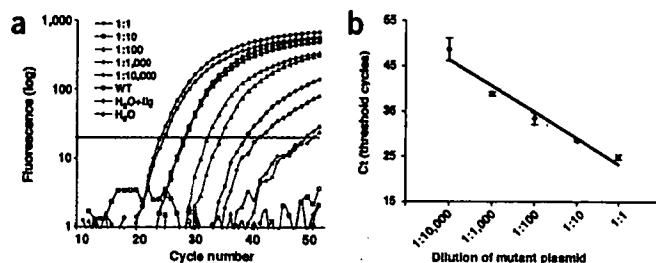
We then conducted LigAmp assays using *KRAS2*-amplified DNA from the pancreatic duct juice samples. First, we confirmed that the level of wild-type DNA was roughly equivalent in the four samples using a wild-type LigAmp reaction (data not shown). We then tested whether we could detect *KRAS2* mutations in the pancreatic duct juice samples. With oligonucleotides specific for the G35A (GAT) mutation (Fig. 3b), the two tumors known to

contain that mutation amplified first (PJ7, blue and PJ70, red). The sample that contained the G35T (GTT) mutation (PJ89, green) amplified between the samples with G35A and the control DNA. Detection of the G35A mutation in this sample could reflect either intratumor clonal heterogeneity of *KRAS2* mutations<sup>23</sup> or the presence of high-grade PanINs that contributed DNA with the G35A mutation to the pancreatic duct juice. The sample with wild-type alleles (PJ66, gold) overlapped with the control DNA (no G35A detected). With oligonucleotides specific for the G35T (GTT) mutation (Fig. 3c), the pancreatic duct juice from the *KRAS2* G35T-bearing tumor (PJ89, green) was amplified before the others. The two tumors with G35A mutations (PJ7 and PJ70) were detected at a lower level, and the wild-type tumor (PJ66) overlapped with the control DNA. In both experiments, a minimal amount of nonspecific amplification was seen with the control DNA (Fig. 3b,c). The presence of the minor mutant species in the pancreatic duct juice samples (GTT in PJ7, GTT in PJ70, and GAT in PJ89) were independently confirmed using *Bst*NI restriction enzyme digestion of wild-type alleles<sup>24</sup> followed by AS-PCR detection of the GAT and GTT mutations. We further confirmed the presence of the minor mutations in samples PJ70 and PJ89 by cloning the *Bst*NI-refractory PCR products and DNA sequencing (data not shown).

### Detection of the K103N mutation in HIV-1 plasmids

We also used the LigAmp assay to detect the K103N HIV-1 drug-resistance mutation. Both the mutant and wild-type upstream oligonucleotides contained an additional base substitution (A→G) at the third base from the 3' terminus of the upstream oligonucleotide (Fig. 5a; the first base of codon 103, underlined G), to enhance specificity of the oligonucleotides for their respective templates. We prepared mixtures of plasmids with wild-type (K = AAA) or mutant (N = AAC) sequences at codon 103, and then conducted ligation using mutant upstream and common downstream oligonucleotides (Fig. 4). Q-PCR was carried out using M13 primers and a *lacZ* probe. The K103N mutation was detected at a dilution of 1:10,000 (0.01%, Fig. 4a); detection was linear over the full range of dilutions tested ( $r^2 = 0.96$ , least-squares analysis, Fig. 4b). Experiments done with a wild-type





**Figure 4 | Detection of the HIV-1 K103N mutation in plasmid mixtures.**

(a) Representative Q-PCR amplification curves (in duplicate) of K103N mutant plasmid serially diluted with wild-type HIV-1 plasmid. WT, Wild-type plasmid alone. Controls ( $H_2O$  + lig and  $H_2O$ ) are described in the legend for Figure 2. (b) The mean Ct values (four independent assays) were plotted against the relative concentration of mutant plasmid. Error bars represent 1 s.d.

upstream oligonucleotide confirmed the specificity of the assay (data not shown).

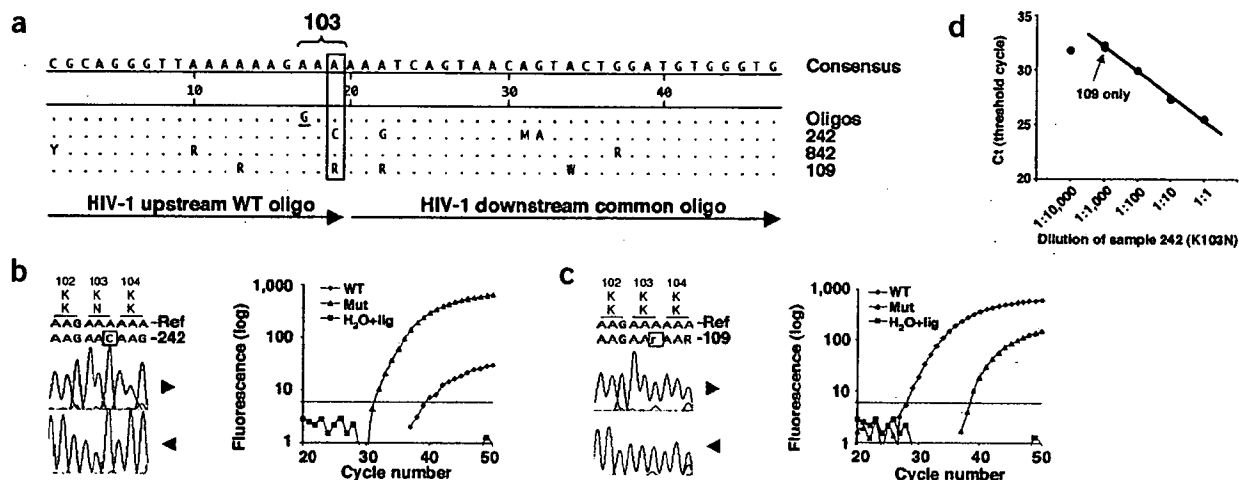
### Detection of the K103N mutation in plasma from individuals infected with HIV-1

We then used the LigAmp assay to detect K103N in plasma samples from individuals infected with HIV-1. We first conducted HIV-1 genotyping (population sequencing) to detect the major population of viruses in each sample. At codon 103, subject 242 had N = AAC (mutant, Fig. 5b), subject 109 had K = AAR = AAA/G (wild

type, Fig. 5c) and subject 842 had K = AAA (wild type, data not shown). The HIV-1 sequences in the samples differed from the sequences of the LigAmp oligonucleotides at three or four positions (Fig. 5a), reflecting the natural genetic diversity of HIV-1 in infected individuals. Analysis of sample 242 with mutant and wild-type upstream oligonucleotides yielded Ct values of 31.6 and 38.9, respectively (Fig. 5b, right). This indicates that viruses with the K103N mutation represent the major viral population, and that a small amount of wild-type HIV-1 is also present. Analysis of samples 109 (Fig. 5c, right) and 842 (data not shown) yielded lower Ct with the wild-type oligonucleotide than with the mutant oligonucleotide, consistent with the genotyping results. In both samples, Q-PCR achieved threshold detection using the mutant upstream oligonucleotide; this was not the case when a pure wild-type plasmid was tested (Fig. 4). This suggests that the K103N mutation was minimally present in both samples. The difference in the Ct values for the mutant versus the wild-type oligonucleotides was 8.3 for subject 842 and 10.3 for subject 109.

### Detection of the K103N mutation in a plasma dilution panel

We conducted additional experiments using samples prepared by serially diluting plasma from subject 242 (mostly mutant) with plasma from subject 109 (mostly wild type). Each sample was independently subjected to HIV-1 RNA extraction, reverse transcription and PCR amplification before analysis. LigAmp detected the K103N mutation in the mixtures at a dilution of 1:1,000 (0.1%,  $r^2 = 0.998$ , least-squares analysis, Fig. 5d). We obtained similar Ct



**Figure 5 | Population sequencing and LigAmp analysis of HIV-1 in plasma samples.** (a) Plasma HIV-1 from subjects 242, 842 and 109 were sequenced with the ViroSeq system (population sequencing). Those sequences were aligned with sequences of the HIV-1 upstream WT and downstream common oligonucleotides (arrows) using MegAlign (DNASTar). A consensus sequence is shown at the top. Nucleotides at the third position of codon 103 (bracket) are boxed. An A→G substitution at the first base of codon 103 in the upstream wild-type and mutant oligonucleotides (underlined) enhances specificity of the ligation reaction. Dots indicate nucleotides that match the consensus sequence. Nucleotide mixtures are indicated using IUB codes. (b,c) On the left, plasma HIV-1 was analyzed using the ViroSeq system. Electropherograms show sequences near codon 103 in HIV-1 reverse transcriptase. Arrowheads indicate the orientation of ViroSeq sequencing primers. The sequence of the HXB2 reference strain ('Ref') is shown above the sequence of each sample. Amino acids encoded by the reference sequence (above) and the sample sequence (below) are shown at the top of each panel. The nucleotide at the third position of codon 103 is boxed. A mixture of nucleotides (A and G = R) is present at this position in the sequence from subject 109 in c; the lower-case designation (r) indicates that the nucleotide sequence was edited manually. On the right, Q-PCR amplification curves from subjects 242 and 109, respectively. Curves were generated using either the mutant (Mut, red) or wild-type (WT, blue) upstream oligonucleotide. (d) Plasma from subject 242 (mostly HIV-1 with the K103N mutation) was serially diluted with plasma from subject 109 (mostly wild-type HIV-1). The percentage of plasma from subject 242 in the samples was 100%, 10%, 1%, 0.1%, 0.01% and 0%, all at 50,000 copies/ml HIV-1 RNA. DNA was amplified from the plasma mixtures using the ViroSeq system. LigAmp was conducted using the upstream mutant oligonucleotide for detection of K103N. Ct values were plotted against the dilution of sample 242 (red dots and line). The Ct of the sample of plasma from subject 109 only (blue dot, arrow) was similar to the Ct obtained with a 1:1,000 dilution of plasma from subject 242.

using the 1:1,000 dilution, further 10-fold dilutions and plasma from subject 109 alone (Fig. 5d), suggesting that plasma from subject 109 contained a minor population of viruses with the K103N mutation (~0.1%).

## DISCUSSION

We show here that the LigAmp assay can detect single-base mutations in the presence of a large excess of wild-type DNA. We easily achieved a sensitivity of  $10^{-3}$ – $10^{-4}$  for detection of point mutations in genomic DNA and HIV cDNA. Several variables could potentially interfere with the sensitivity of LigAmp for analysis of clinical samples. Mutations present in very small amounts could be lost through sampling error or 'bottlenecking.' Some samples might also contain DNA ligase inhibitors<sup>25,26</sup>. Mutation detection could also be hampered by biased amplification during the sample prep PCR or Q-PCR. For these reasons, the sensitivities we obtained using control reagents may be better than those obtained when the system is validated using blinded panels and clinical specimens. The type of template used may also influence the efficiency of the LigAmp assay. Ct values are slightly lower when PCR products are used for templates, as opposed to the plasmids used to generate standard curves, and when the PCR products contain dUTP. Therefore, samples used to generate standard curves should be obtained in the same way as the test samples. Accurate quantification of template DNA is also important; the Q-PCR reaction is quite sensitive to template concentration if a single oligonucleotide (such as mutant) is used. When template concentration is carefully controlled, LigAmp results correlate closely with the percentage of mutant DNA (Figs. 2b and 4b). Use of an internal standard with each reaction may also be helpful.

Although the specificity of LigAmp is quite high, we observed small amounts of nonspecific amplification of wild-type templates in some experiments (Figs. 2a and 3b,c). Nonspecific signals could arise during ligation, reflecting either minimal ligation of oligonucleotides despite terminal mismatches with the template, or template-independent oligonucleotide ligation<sup>27</sup>. Alternatively, nonspecific signals could be generated during Q-PCR. Unligated oligonucleotides were not removed before Q-PCR, and these might extend on mismatched wild-type templates during Q-PCR by *Taq* polymerase. M13 reverse primers could then extend from unligated downstream oligonucleotides, producing a complementary strand. Because the extended products are complementary to each other, nonspecific amplification might then occur. Experiments are in progress to identify variables contributing to nonspecific signals in the assay.

LigAmp requires the use of high-quality oligonucleotides. Synthetic errors in the oligonucleotides could influence sensitivity and specificity of the assay. It is important to obtain oligonucleotides from a reliable source and to test new batches of primers with standardized reagents and controls before using them for analysis. We used gel-purified oligonucleotides for ligation, and it is not clear whether the results would have been as good using unpurified oligonucleotides. Other variables, such as pipetting error or unequal thermal cycling temperatures during ligation or Q-PCR, could potentially lead to inaccurate results. Therefore, we recommend analyzing specimens in duplicate or triplicate experiments to ensure reproducibility.

Other methods for sensitive detection of point mutations are generally more complex and less quantitative than the LigAmp

assay. Also, because no unique sequence context (for example, a restriction enzyme site) is required, and because universal primers and probes are used in the Q-PCR step, LigAmp should be easily adapted for detection of any single-base difference. We present preliminary data indicating that LigAmp can also be carried out as a multiplex reaction for simultaneous detection of mutant and wild-type DNA or for simultaneous detection of different mutations. Other investigators have shown that it is possible to ligate simultaneously as many as 40 oligonucleotide pairs<sup>28</sup>. However, additional experiments are required to evaluate the effect of multiplexing on sensitivity.

Accurate quantification of mutant DNA by LigAmp may prove useful for early detection of pancreatic cancer, because the level of *KRAS2* mutant DNA in pancreatic duct juice may differ in patients with benign versus malignant lesions. Detection of *KRAS2* in combination with other mutations such as *p53* may increase both the sensitivity and specificity of LigAmp for detection of pancreatic cancer and its precursor lesions.

Analysis of mutations in HIV-1 poses unique challenges because of viral genetic diversity. We tested samples from three subjects using a single oligonucleotide set. None of the subjects had other drug resistance mutations near K103N, but there were numerous nucleotide differences in the oligonucleotide binding regions. Those differences did not seem to hamper detection of the K103N mutation, however. Analysis of wild-type-K103N plasmid pairs from other individuals indicates that nucleotide diversity near K103N can be overcome by extending the length of the oligonucleotides and lowering the ligation temperature (data not shown). Different primer sets may be needed for different HIV subtypes. Some HIV-1 variants have other mutations at codon 103 (for example, K103E, K103R and K103T). Specific oligonucleotides would be needed to detect each of those unusual mutations.

It is difficult to define the limit of sensitivity of LigAmp for HIV-1 mutation detection using clinical samples, because many samples that are genotypically wild type are thought to harbor minority variants with drug resistance mutations<sup>29,30</sup>. Using plasmid mixtures, we detected K103N at 0.01%. We also detected K103N in small amounts in two patients who had only the wild-type sequence detected with an FDA-cleared genotyping assay. A dilution experiment suggested that K103N was present at ~0.1% in one of those patients. We are presently optimizing LigAmp for quantification of HIV-1 drug-resistant variants in plasma samples. Additional studies are needed to compare the efficacy of different HIV-1 minority variant assays in different clinical settings. LigAmp can be conducted using PCR products remaining from routine HIV-1 genotyping. Because PCR products are used at low concentrations, numerous mutations can be analyzed using PCR products remaining after genotyping a single 0.5-ml plasma sample.

LigAmp may also have applications in genetics, including prenatal diagnosis (for example, multiplex detection of common *CFTR* mutations)<sup>31</sup>. Using LigAmp, it may be possible to detect point mutations in fetal cells circulating in maternal peripheral blood, avoiding the need for amniocentesis or chorionic villous sampling procedures<sup>32,33</sup>.

In summary, LigAmp is a universal point mutation detection strategy that is highly sensitive and quantitative. This report presents proof-of-principle experiments that show some features of this assay. Additional validation is needed to identify

preanalytical and analytical variables that influence assay performance, and to define the sensitivity, specificity, accuracy and precision of the assay for various clinical applications.

## METHODS

**Experimental review.** We obtained appropriate institutional approval for all experiments involving human subjects.

**Genomic DNA isolation.** We isolated genomic DNA using the DNeasy Tissue Kit (Qiagen)<sup>34</sup>.

**KRAS2 sequencing.** We amplified the KRAS2 locus by PCR using the following M13-tailed primers (M13 tails underlined): 5'-GTAAAACGACGGCCAGG-GAGAGAGGCCTGCTGAAAA-3' and 5'-CAGGAAACAGCTATGACT-TGGATCATATTCGTCCACA-3'. We carried out sequencing using M13 primers, the BigDye Terminator 3.1 Cycle Sequencing Kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

**Population sequencing of plasma HIV-1.** We sequenced plasma samples diluted at 100,000 copies/ml HIV-1 RNA (using plasma from an individual without HIV-1 infection) with the ViroSeq HIV-1 Genotyping System (Celera Diagnostics). Using this system, we extracted and reverse transcribed HIV-1 RNA. A 40-cycle PCR yielded a 1.8-kb PCR product that is purified and sequenced (BigDye; Applied Biosystems) with seven different primers using an ABI Prism 3100 Genetic Analyzer.

**LigAmp oligonucleotides and probes.** We purchased gel-purified oligonucleotides and M13 primers from Invitrogen, and lacZ and 16S rDNA Taqman probes from Integrated DNA Technology (see **Supplementary Table** online for sequences).

**KRAS2 and p53 oligonucleotide ligation.** Ligation was conducted directly on genomic DNA from cultured cells. For pancreatic duct juice samples, ligation was done using 60 pg of PCR-amplified KRAS2 DNA (described earlier). We incubated DNA samples with 1 pmol of each oligonucleotide (either a mutant or wild-type upstream oligonucleotide and a downstream common oligonucleotide) and 4 U Pfu DNA ligase in 1× Pfu ligase buffer (Stratagene). We denatured samples at 95 °C for 3 min and then incubated them for 90 two-step cycles of 95 °C for 30 s alternating with 65 °C for 4 min. For simultaneous detection of mutant and wild-type KRAS2, we included both upstream oligonucleotides in the reaction; the concentration of the wild-type oligonucleotide was reduced to 10<sup>-5</sup> pmol. For simultaneous detection of KRAS2 and p53 mutations, we included both upstream and downstream oligonucleotides at 1 pmol.

**HIV-1 K103N oligonucleotide ligation.** John Mellors (University of Pittsburgh) provided HIV-1 clones with and without the K103N mutation. We amplified DNA from these molecular clones with the ViroSeq system and cloned the amplified DNA using the TOPO TA Cloning Kit (Invitrogen) to generate plasmids containing wild-type and mutant inserts. We isolated these plasmids and sequenced them as described<sup>35</sup>. Ligation was done using either 10 pg of plasmid template or 100 pg of plasma-derived ViroSeq PCR products as described earlier, except that the upstream oligonucleotide concentration was 2 pmol.

**Q-PCR.** Q-PCR was conducted using a SmartCycler (Cepheid). Each 25-μl reaction contained 5 pmol forward and 5 pmol reverse M13 primers, 6 μl of the unpurified ligation reaction, 12.5 μl platinum Quantitative PCR SuperMix-UDG (Invitrogen) and 2.5 pmol of lacZ and/or 16S rDNA probes. We preincubated PCR reactions at 50 °C for 2 min and 95 °C for 2 min, and then subjected them to 50 two-step cycles of 95 °C for 10 s alternating with 64 °C for 20 s. We manually set the Ct in the middle of the linear range of the amplification curves (log scale).

*Note: Supplementary information is available on the Nature Methods website.*

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## COMPETING INTERESTS STATEMENT

The authors declare competing financial interests; see the Nature Methods website for details.

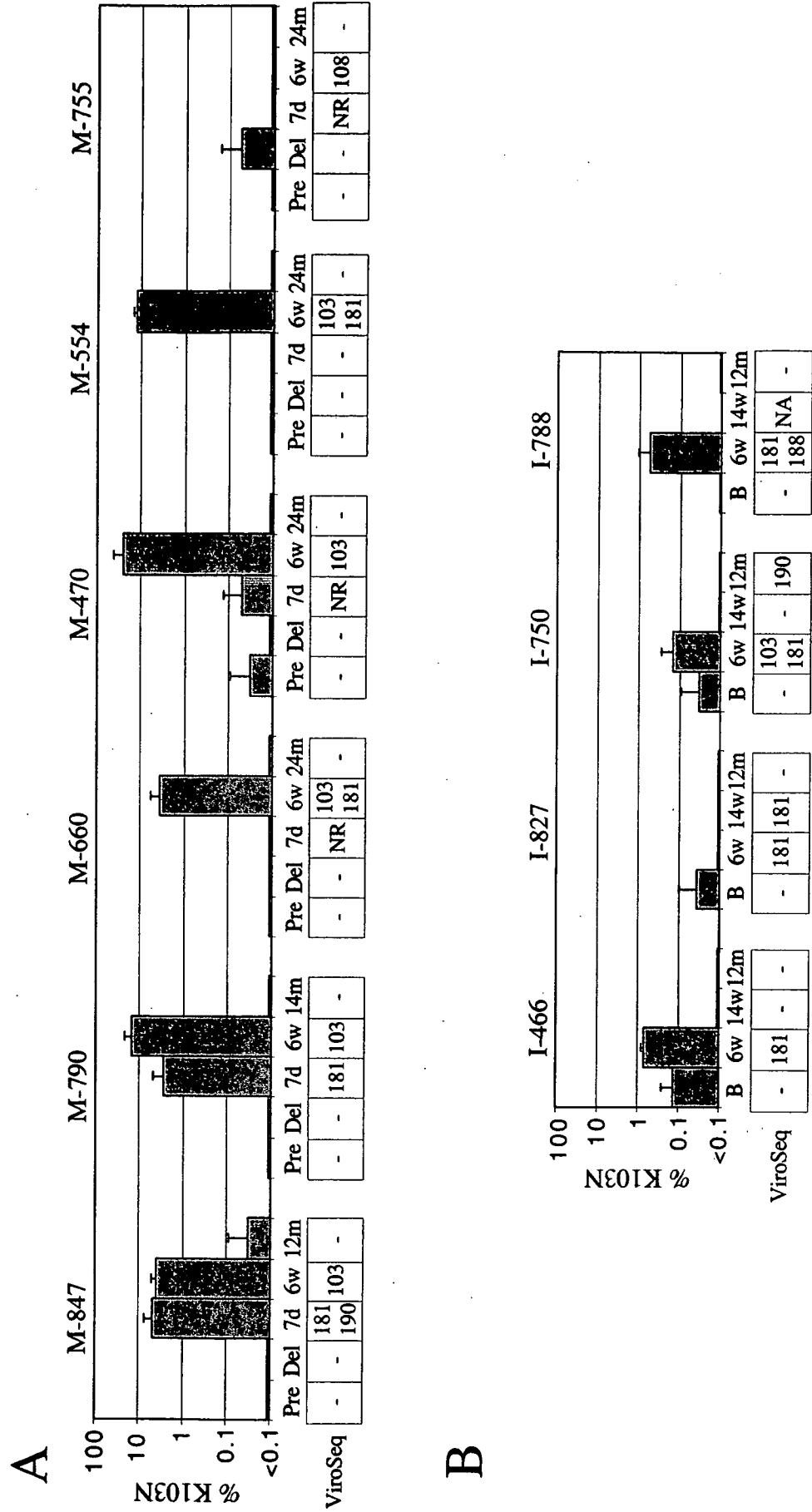
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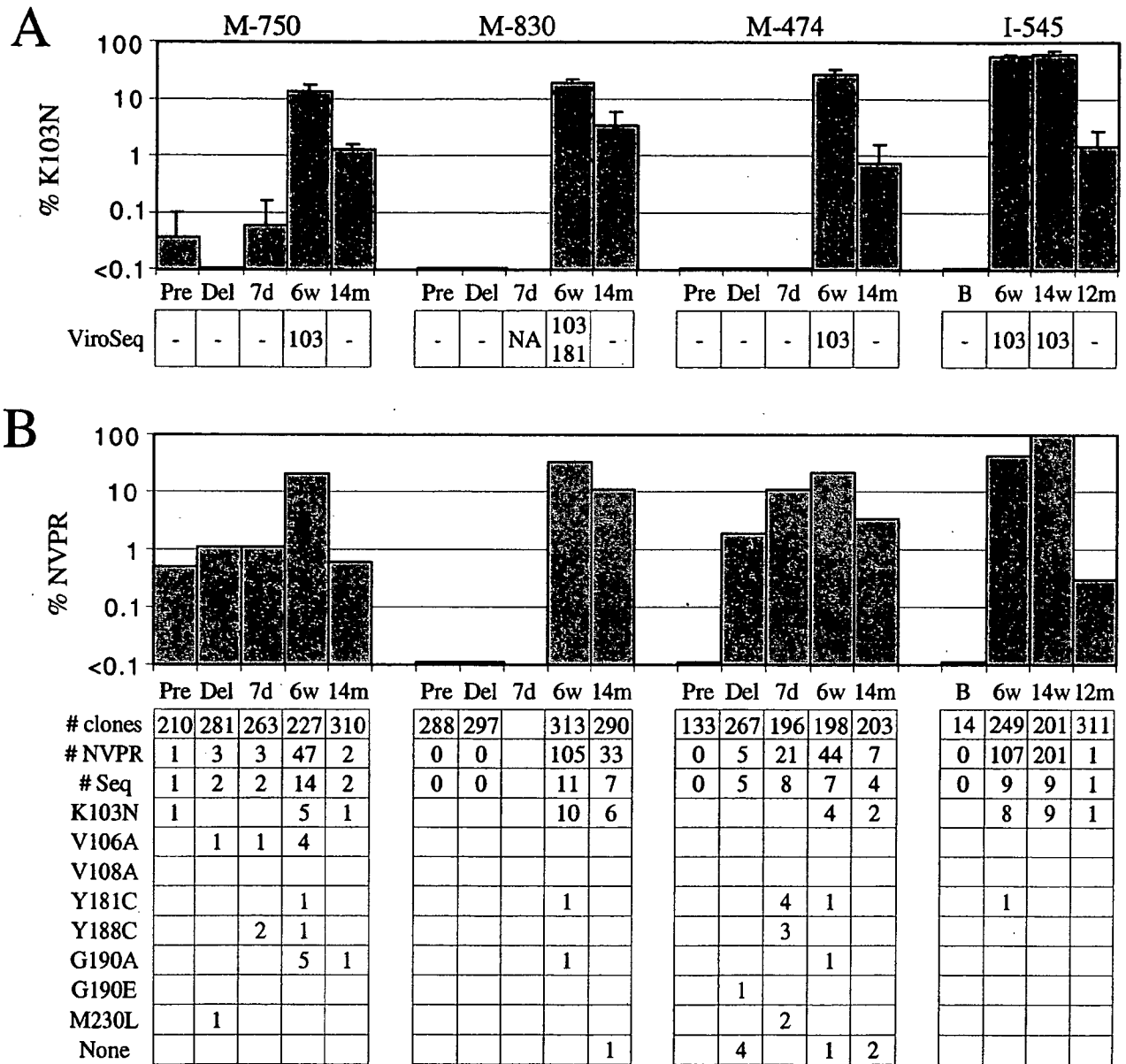
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Flys et al, Figure 1

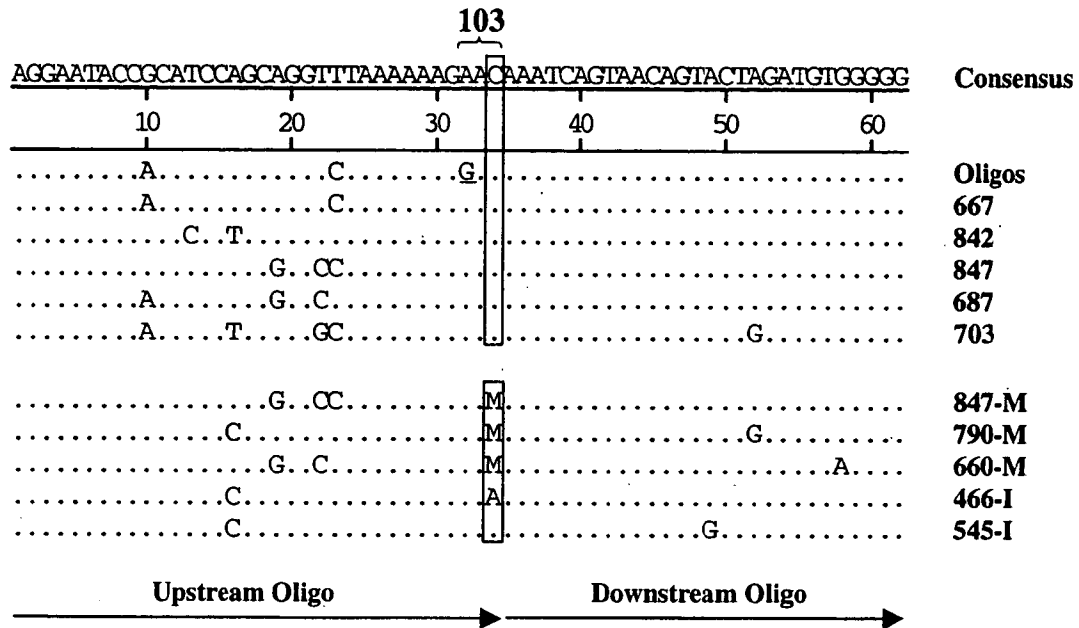


Flys et al, Figure 2

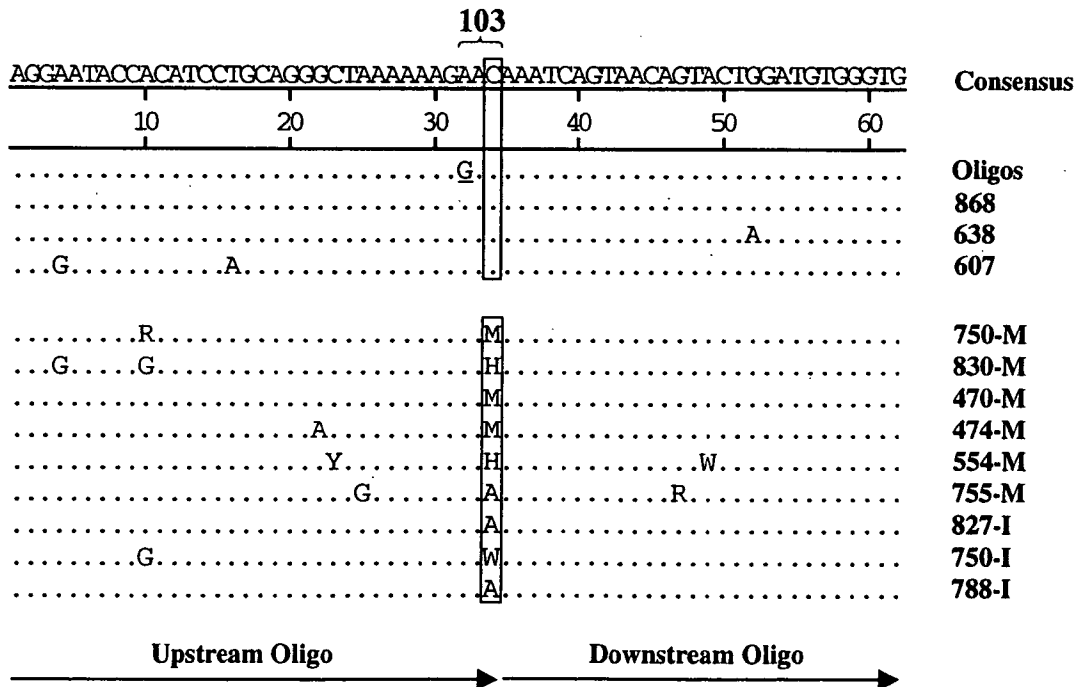


Flys et al, Figure 3

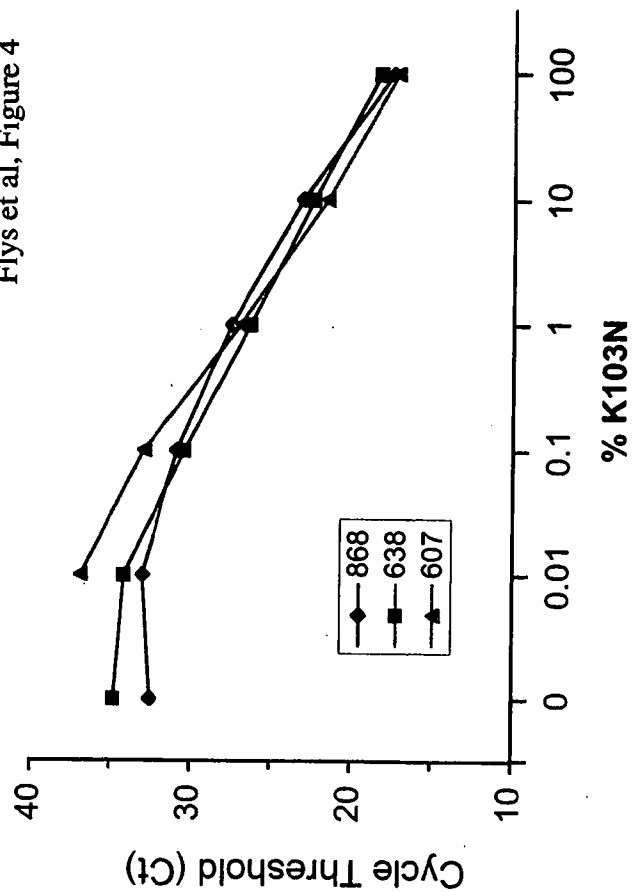
**A**



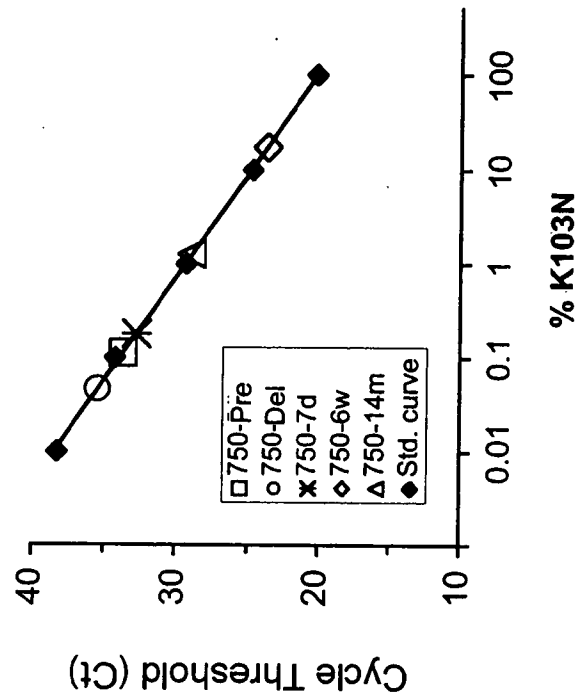
**B**



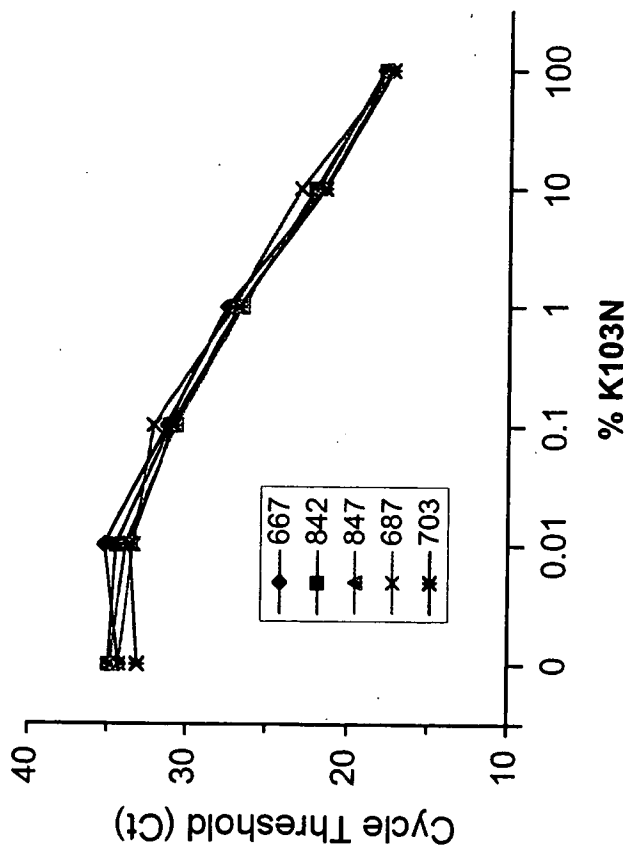
**B**



**D**



**A**



**C**

